J. Phycol. **57,** 279–294 (2021)

DOI: 10.1111/jpy.13088-20-124

USING RAD-SEQ TO DEVELOP SEX-LINKED MARKERS IN A HAPLODIPLONTIC ALGA[1]

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For many taxa, including isomorphic haplodiplontic macroalgae, determining sex and ploidy is challenging, thereby limiting the scope of some population demographic and genetic studies. Here, we used double-digest restriction site-associated DNA sequencing (ddRAD-seq) to identify sex-linked molecular markers in the widespread red alga *Agarophyton vermiculophyllum*. In the ddRAD-seq library, we included 10 female gametophytes, 10 male gametophytes, and 16 tetrasporophytes from one native and one non-native site ($N = 40$ gametophytes and $N = 32$ tetrasporophytes total). We identified seven putatively female-linked and 19 putatively male-linked sequences. Four female- and eight male-linked markers amplified in all three life cycle stages. Using one female- and one male-linked marker that were sex-specific, we developed a duplex PCR and tested the efficacy of this assay on a subset of thalli sampled at two sites in the non-native range. We confirmed ploidy based on the visual observation of reproductive structures and previous microsatellite genotyping at 10 polymorphic loci. For 32 vegetative thalli, we were able to assign sex and confirm ploidy in these previously genotyped thalli. These markers will be integral to ongoing studies of *A. vermiculophyllum* invasion. We discuss the utility of RAD-seq over other approaches previously used, such as RAPDs (random amplified polymorphic DNA), for future work designing sex-linked markers in other haplodiplontic macroalgae for which genomes are lacking.

Key index words: ***Agarophyton vermiculophyllum*****; diploid; haploid; *Gracilaria vermiculophylla*; life cycle; PCR; seaweed; single nucleotide polymorphism**

***Abbreviations*: RAPD, random amplified polymorphic DNA; RAD-seq, restriction site-associated DNA sequencing; ddRAD-seq, double-digest restriction site-associated DNA sequencing; bp, base pair; SDR, sex-determining region; RFLP, restriction fragment length polymorphism**

Eukaryotic life cycles can be characterized as linked cycles of growth and reproduction joined together through the processes of meiosis and syngamy (Bell 1994). Sexual reproduction, therefore, results in the alternation between haploid and diploid life cycle stages. While this alternation is an ancestral and highly conserved process across eukaryotes (Speijer et al. 2015), our understanding of the mechanisms and processes that generate and maintain life cycle variation is limited. For example,

[1]Received 3 June 2020. Accepted 5 October 2020.
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Editorial Responsibility: M. Oliveira (Associate Editor)

theoretical and empirical studies have focused on the genetic benefits of haploidy (e.g., purging of deleterious mutations) versus diploidy (e.g., masking of deleterious mutations; reviewed in Valero et al. 1992, Mable and Otto 1998). Yet, for haplodiplontic taxa, the genetic advantages of haploidy and diploidy cannot alone explain the maintenance of somatic development in both haploid and diploid stages in the same life cycle. However, haplodiplontic life cycles are stabilized over evolutionary timescales by ecological differentiation, even for the morphologically similar haploid and diploid stages found in isomorphic life cycles (Hughes and Otto 1999).

The niche differentiation hypothesis has not been widely tested across the macroalgae (see reviews by Thornber 2006, Krueger-Hadfield 2020; and text and references in Scott and Rescan 2017, Krueger-Hadfield and Ryan 2020). One impediment to testing this hypothesis is the reliance on reproductive material with which to distinguish morphologically similar dioecious, haploid gametophytes, and diploid sporophytes (Fig. 1). At any one time, many natural macroalgal populations are composed largely of vegetative thalli (e.g., Prince and Kingsbury 1973, Dyck et al. 1985, Lazo et al. 1989, Kain and Destombe 1995, Shaughnessy et al. 1996, Mudge and Scrosati 2003, Krueger-Hadfield et al. 2013). While in some taxa, such as *Chondrus crispus* (Lazo et al. 1989), chemical tests are available with which to distinguish gametophytes from sporophytes, many taxa do not have reliable ways with which to determine ploidy in the absence of reproductive individuals. Further, the resorcinol test in a species such as *C. crispus* may enable the determination of ploidy, but the determination of sex is not possible. Instead, studies must still rely on reproductive gametophytes (see also Lipinska et al. 2020), which is further complicated as males are often exceedingly hard to identify (e.g., Tveter-Gallagher et al. 1980, Krueger-Hadfield et al. 2015). Recent population genetic studies (e.g., Engel et al. 2004, Guillemin et al. 2008a, Krueger-Hadfield et al. 2013, 2016) have used polymorphic molecular markers, such as microsatellites, in order to determine ploidy where "fixed homozygotes" (i.e., one allele per locus) at all genotyped loci are interpreted as haploid and any thallus with at least one heterozygous locus is considered diploid. While this method does permit the determination of ploidy in taxa without chemical tests, few microsatellite loci have been linked to sex (but see female-linked microsatellite-based markers in Liu et al. 2009, Shan and Pang, 2010, Gu et al. 2014).

Not only does the lack of a diagnostic tool for sex determination limit the types of demographic studies that can be undertaken, but it also prevents investigations into the evolutionary ecology of haplodiplontic macroalgae. For example, Guillemin et al. (2013) found that vegetative thalli of *Agarophyton chilense* (reported as *Gracilaria chilensis*) grew faster than reproductive thalli. While unsurprising, differences in reproductive state may also explain patterns of rapid growth in farmed populations of *A. chilense*, where thalli are overwhelmingly diploid and are often vegetative (Guillemin et al. 2008a). Recently, Krueger-Hadfield and Ryan (2020) found decreased growth rates in reproductive females compared to male and tetrasporophytic *A. vermiculophyllum* in response to nutrient-limited and nutrient-replete conditions, but these were site-specific. Red algal female gametophytes invest a substantial amount of energy in nurturing developing zygotes and carpospores in the carposporophyte (Searles 1980), which may explain these results (see also Lees et al. 2018). Thus, ecophysiological studies based on reproductive material, often by necessity, may obscure differences among life cycle stages in response to different biotic or abiotic factors.

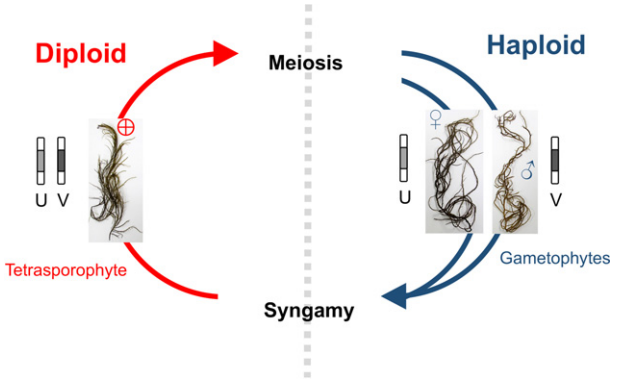

FIG 1. Eukaryotic sexual life cycles involve the alternation between haploid and diploid stages as a consequence of meiosis and syngamy (i.e., fusion of haploid gametes), respectively. For haplodiplontic taxa, such as *Agarophyton vermiculophyllum*, sex is determined in the haploid stage in dioecious gametophytes (i.e., genetically and morphologically distinct males [V] and females [U] produce gametes; adapted from Coelho et al. 2018; photo credit: S.A. Krueger-Hadfield). [Color figure can be viewed at wileyonlinelibrary.com]

In haplodiplontic taxa, such as macroalgae and mosses, sex is genetically determined *after* meiosis in the haploid stage (Fig. 1; see reviews by Bachtrog et al. 2011, Coelho et al. 2018). The most detailed work on macroalgal sex determination systems is found across the brown algae (e.g., Lipinska et al. 2015), most notably in detail in the brown algal model *Ectocarpus* (e.g., Ahmed et al. 2014). The UV sexual system has unique evolutionary and genetic characteristics that have no analog in the more commonly studied diploid sexual systems XY (e.g., humans) and ZW (e.g., birds; see Ahmed et al. 2014, Coelho et al. 2018, among others, for reviews on the evolutionary and genetic consequences of UV sex determination). For example, female (U) and male (V) sex-determining regions function in separate haploid male and haploid female individuals and, as a consequence, may be subject to similar evolutionary pressures unlike XY/ZW systems in which there is a heterogametic sex (Bull 1978).

Moreover, the UV system affords an opportunity to generate sex-linked markers that enable the characterization of sex and ploidy using simple PCR assays that are not based solely on presence/absence. Markers developed from regions that are male-linked (V) and female-linked (U) will amplify in males and females, respectively, but not the opposite sex, and sporophytes will have both U- and V-linked markers (Figs. 1, 2a). Simple PCR-based assays can rapidly assess the proportions of female gametophytes, male gametophytes, and sporophytes in populations where gametophytes have a single, sex-linked band and sporophytes have both bands (Fig. 2a).

To date, a handful of sex-linked markers have been developed in the three lineages of macroalgae, and the few taxa for which markers are available are either important models for genetics (e.g., *Ectocarpus*; Ahmed et al. 2014) or commercially important taxa, such as *Agarophyton chilense* (Guillemin et al. 2012) or kelps (Lipinska et al. 2015). Methods for sex-linked marker development in macroalgae have used bulk segregant analysis of RAPDs (random amplified polymorphic DNA; Martinez et al. 1999, Guillemin et al. 2012) or, more recently, have made use of available genomes (Ahmed et al. 2014, Lipinska et al. 2015).

More recently, restriction-associated DNA sequencing, or RAD-seq, has been used to characterize markers linked to sex-determining regions in diverse taxa, such as moths (Baxter et al. 2011), fish (zebrafish: Anderson et al. 2012; Atlantic halibut: Palaiokostas et al. 2013), and a parasitic copepod (Carmichael et al. 2013). RAD-seq is a powerful molecular tool for characterizing sex-linked markers because (i) RAD-seq sequence data can easily be turned into a simple PCR assay, unlike RAPD approaches that require subsequent cloning steps for primer design (see as an example Guillemin et al. 2012), and (ii) sequenced genomes are not required for RAD-seq studies (Gamble 2016). Few macroalgae have fully sequenced genomes (but see, Cock et al. 2010, Collén et al. 2013, Brawley et al. 2017). Previous studies used RAD-seq in combination with linkage maps from progeny arrays (e.g., Baxter et al. 2011, Anderson et al. 2012), whereas

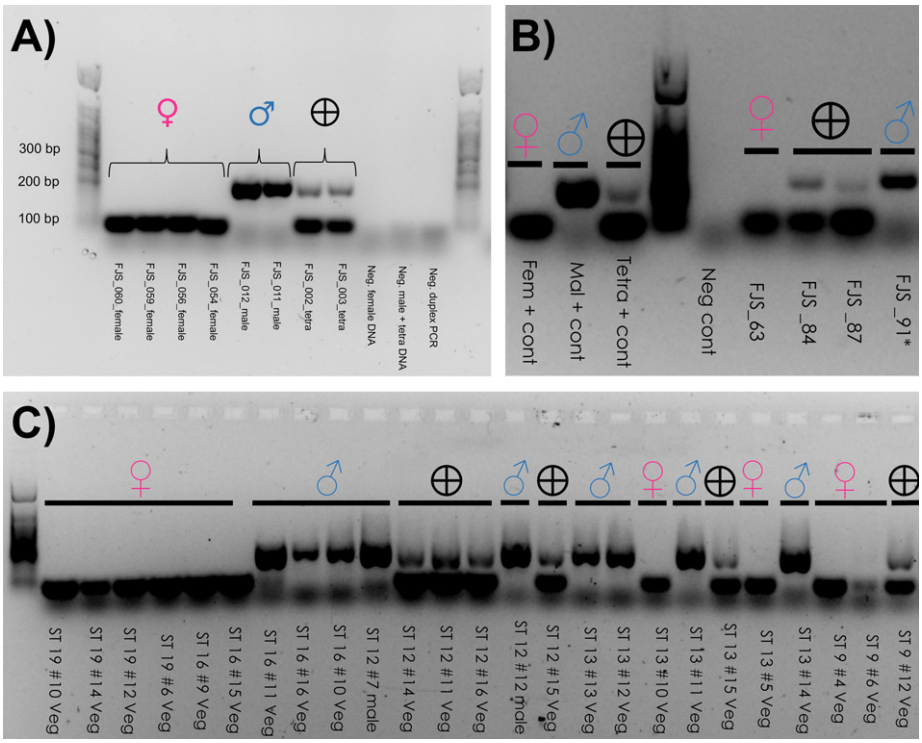

FIG 2. Example gels showing marker development and efficacy tests. All thalli were sampled from Fort Johnson, SC in 2015 or 2016. A 50-bp ladder was used to size PCR products. ♀, female gametophyte; ♂, male gametophyte; ⊕, tetrasporophyte; veg, vegetative. (a) Duplex PCR amplification of the female-linked and male-linked with Chelex-extracted DNA of ~ 1cm of thallus. Four female gametophytes, two male gametophytes, two tetrasporophytes, and negative controls (including DNA extraction with PCR mix and PCR mix negatives). The four female thalli were sampled in 2016 (see Lees et al. 2018). (b) Example gel with positive and negative controls and four thalli from 2015 that were identified as vegetative (FJS_63, FJS_84, FJS_87) and one as a tetrasporophyte (FJS_91). * FJS_91 was identified as haploid based on 10 microsatellites (Krueger-Hadfield et al. 2017), and the current study determined it to be a male gametophyte. The other three thalli were not included in previous microsatellite genotyping efforts. (c) Sex determination of the 24 thalli sampled from PVC settlement posts (see also Table 3). ST refers to the PVC settlement post number followed by the thallus number that was assigned when thalli were subsampled and placed into silica gel. The reproductive state of each thallus is also provided (veg, vegetative). [Color figure can be viewed at wileyonlinelibrary.com]

Gamble and Zarkower (2014) developed a workflow based on accurately sexed individuals followed by bioinformatic identification of sex-specific markers that can be validated by PCR without relying on progeny arrays. Thus, for macroalgae, with or without a reference genome, RAD-seq approaches may be a more efficient way to search for sex-linked markers.

In this study, we applied a double-digest RAD-seq (ddRAD-seq) approach in order to characterize molecular markers linked to sex determination in *Agarophyton vermiculophyllum* (synonym *Gracilaria vermiculophylla*). This red macroalga has invaded nearly every temperate estuary in the Northern Hemisphere over the last ~100 y (Bellorin et al. 2004, Rueness 2005, Guillemin et al. 2008b, Kim et al. 2010, Krueger-Hadfield et al. 2017, 2018). Gametophytes and tetrasporophytes are morphologically indistinguishable when they are not reproductive and many populations include a majority of vegetative thalli (Krueger-Hadfield et al. 2016, 2017, 2018). The *A. vermiculophyllum* invasion has been facilitated, in part, by a propensity for asexual reproduction through thallus fragmentation (Krueger-Hadfield et al. 2016). In both the native and non-native ranges, tetrasporophytes consistently dominate free-floating populations where thalli drift and fragment in soft-sediment habitats without abundant hard substratum (Krueger-Hadfield et al. 2016, 2017). *Agarophyton vermiculophyllum* is emerging as a model system with which to address hypotheses central to our understanding of biological invasions, including adaptation (e.g., Hammann et al. 2016, Wang et al. 2017a, Sotka et al. 2018), novel mutualisms (Thomsen and McGlathery 2005, Kollars et al. 2016), escape from herbivory (e.g., Hammann et al. 2013, Bippus et al. 2018), and fouling by microbes and other epiphytes (e.g., Saha et al. 2016, Wang et al. 2017b, Bonthond et al. 2020). In addition, recent studies have demonstrated the necessity of understanding the evolutionary ecology of haplodiplontic life cycles in the context of biological invasions (Lees et al. 2018, Krueger-Hadfield and Ryan 2020) as there are unique consequences resulting from disruptions to the life cycle, such as the loss of a free-living ploidy stage as a result of asexual reproduction (see review by Krueger-Hadfield 2020).

We developed sex-linked markers from reproductive thalli sampled in the native and non-native ranges. We tested the male- and female-linked markers on a subset of thalli that were sampled from sporic recruits from a non-native site. Previous work at this site demonstrated the presence of female gametophytes, male gametophytes, and tetrasporophytes through the combination of morphological identification of reproductive material and microsatellite genotyping confirming ploidy on settlement posts (Lees et al. 2018). We identified one female- and one male-linked marker from which we developed a duplex PCR assay to determine ploidy and/or sex.

MATERIALS AND METHODS

Sample collection. We collected reproductive and nonreproductive thalli from one site in the native range (Mangoku-ura, Japan) and two sites in the non-native range (Fort Johnson, Charleston, South Carolina and Wachapreague, Virginia, USA; Table 1). Ploidy and sex were determined through observations of reproductive material under a dissecting microscope (40x; see Krueger-Hadfield et al. 2018). If no reproductive structures were observed, then the thallus was considered vegetative. All visible carposporophytes on female thalli were removed prior to preservation in silica gel for subsequent DNA extractions.

DNA extraction. We extracted total genomic DNA using 10 – 15 mg of silica gel-dried tissue and the Nucleospin® 96 plant kit (Macherey-Nagel, Düren, Germany). 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, 2016). DNA was eluted with molecular grade water, heated to 70°C, in two 25 µL elutions.

We also tested the amplification of our markers using an inexpensive DNA extraction method that would enable screening of thalli while using minimal amounts of thallus material. We extracted total genomic DNA from the 53 thalli, including sporic recruits (S.A. Krueger-Hadfield, unpub. data; Lees et al. 2018) and thalli from a previous study on the invasion history of *Agarophyton vermiculophyllum* (Krueger-Hadfield et al. 2017), using 120 µL of a 10% Chelex solution (Bio-Rad Laboratories, Hercules, CA, USA). We used approximately 1 cm of dried algal tissue and heated the Chelex solution at 95°C for 30 min, vortexing intermittently (Walsh et al. 1991). Samples were centrifuged at 13,000 rpm for 10 min, and cleared supernatant was transferred to a new tube for storage and genotyping.

ddRAD-seq library preparation. Due to poor amplification success and the lack of markers identified from a bulk segregant RAPD analysis (see Appendix S1, Table S1 in the Supporting Information), we used a ddRAD-seq approach to identify sex-linked markers (see also Gamble and Zarkower 2014, Gamble et al. 2015, Fowler and Buonaccorsi 2016). As part of a larger genotyping effort (Flanagan 2017; B.A. Flanagan, S.A. Krueger-Hadfield, C.J. Murren, C.C. Nice, A.E. Strand, E.E. Sotka, unpub. data), we constructed a ddRAD-seq genomic library with 10 female gametophytes, 10 male gametophytes, and 16 tetrasporophytes sampled from two sites, Mangoku-ura and Fort Johnson ($N = 36$ thalli per site). All site and thallus collection information are provided in Table 1. In June 2015, at Mangoku-ura, a single thallus was sampled every meter along a transect parallel to the shore as described previously by Krueger-Hadfield et al. (2017). In March 2015, phenotypically male, female, and tetrasporophytic thalli from Fort Johnson were sampled from PVC settlement posts that were deployed on the mudflat between January and April 2014 and were previously described in Lees et al. (2018). We included 16 thalli from Fort Johnson sampled in July 2015 that were either vegetative or tetrasporophytic (Krueger-Hadfield et al. 2017). All thalli included in the ddRAD-seq library were genotyped at 10 microsatellite loci in which ploidy was confirmed (haploid: one allele per locus; diploid: one or two alleles per locus with at least one heterozygous locus; Krueger-Hadfield et al. 2017, Lees et al. 2018). There was one exception, a field-identified tetrasporophyte from Fort Johnson in July 2015 that was determined to be haploid based on the microsatellite multilocus genotype (see Results and Discussion). Microsatellite genotypes are provided in Table S2 in the Supporting Information.

We prepared the genomic library by following protocols in Parchman et al. (2012). Briefly, we digested genomic DNA

TABLE 1. Sample collection and marker testing information for thalli used to characterize sex-linked markers in *Agarophyton vermiculophyllum*. Life cycle stage was based on the morphological observation of reproductive structures. If there were no visible reproductive structures, the thalli were considered vegetative. Sample type describes the type of sampling that was undertaken: invasion history, sampling as part of the invasion history described by Krueger-Hadfield et al. (2017); phenology, sampling as part of a phenological study (S.A. Krueger-Hadfield, E.E. Sotka, C.J. Murren, unpub. data); and PVC recruits, sampling of thalli off of PVC settlement posts (Lees et al. 2018, S.A. Krueger-Hadfield, unpub. data).

Site	Sample type	Date	Collectors	Latitude	Longitude	Number of thalli	Thallus usage
Mangoku-ura, Japan (native range)	Invasion history	25 May 2015	SAKH, CRH, JP, HE	38.418195	141.413173	Female gametophyte: 10 Male gametophyte: 10 Tetrasporophyte: 16	ddRAD-seq library
Fort Johnson, Charleston, South Carolina, USA (non-native range)	PVC recruits	18 March 2015	SAKH			Male gametophyte: 2 Vegetative: 22	Marker efficacy
	PVC recruits	21 April 2015	SAKH			Female gametophyte: 10 Male gametophyte: 10	ddRAD-seq library
	Invasion history	10 July 2015	SAKH, BAF, AG, SJS			Tetrasporophyte: 13[a] Vegetative: 2	ddRAD-seq library
	Invasion history	10 July 2015	SAKH, BAF, AG, SJS			Tetrasporophyte: 6[b] Vegetative: 5[b]	Marker efficacy
Wachapreague, Virginia, USA (non-native range)	Invasion history	2 August 2015	SAKH, BAF, SF	37.619458	-75.669093	Tetrasporophyte: 11 Vegetative: 5	Marker efficacy

[a]One thallus FJS_091 was recorded as a tetrasporophyte based on visual observations at 40x, but based on 10 microsatellites, it was determined to be haploid and removed from other analyses (Krueger-Hadfield et al. 2017; B.A. Flanagan, S.A. Krueger-Hadfield, C.J. Murren, C.C. Nice, A.E. Strand, E.E. Sotka, unpub. data). fjs_091 was determined to be a male gametophyte based on the duplex PCR of fem_o03 and mal_n09.

[b]Five tetrasporophytic and two vegetative thalli included in the marker efficacy testing were included in the ddRAD-seq library and previously genotyped. *Collectors*: SAKH, SA Krueger-Hadfield; CRH, CR Hadfield; JP, J Pocklington; HE, H Endo; SJS, SJ Shainker; BAF, BA Flanagan; AG, A Gaffney; SF, S Fate

with two restriction enzymes, *Eco*RI and *Mse*I, and ligated adaptors with unique 8- to 10-bp barcodes to the digested DNA. Each thallus was assigned a unique barcode. The restriction-ligation products were amplified in two independent PCRs using standard Illumina primers. All amplicons were pooled and sent to the University of Texas Genomic Sequencing and Analysis Facility (Austin, TX, USA). The genomic library was size-selected using the Blue Pippin Prep (Sage Science, Beverly, MA, USA) to isolate the 300- to 500-bp fraction and sequenced as single-end reads on an Illumina HiSeq 2000 and Illumina HiSeq 4000 platforms. Other sequencing information can be found in Flanagan (2017).

Determination of candidate sex-linked contigs. Raw reads were parsed to corresponding individuals based on barcode sequence using a custom perl script. Following previous studies (Nosil et al. 2012, Parchman et al. 2012, Kingston et al. 2017), we performed de novo genome assembly on a subset of 25 million randomly sampled reads from the 40 haploid thalli using *seqman ngnen* software (DNASTAR, Madison, WI, USA) with a minimum match percentage of 92%. To help eliminate paralogs within our de novo assembly, we used only reads from haploids as all variation will occur at different points on the genome (i.e., no heterozygosity), and we assembled our de novo to itself using *seqman ngnen* software with a minimum match percentage of 83% to eliminate potential paralogs. We only retained unassembled contigs, yielding 242,815 contigs. All reads were then aligned to the de novo assembly using *bwa* ver. 0.7.12 (Li and Durbin 2009).

We then used *samtools* 1.3.1 (Li et al. 2009) "idxstat" to generate individual mapping statistics. Using coverage estimates, we queried contigs which had, at minimum, 500 reads present across all 20 males, but reads absent in females, and vice versa. We ensured these putatively sex-linked contigs were found in diploids using a custom *R* (R Core Team 2016) script (see Appendix S2 in the Supporting Information). We extracted sex-linked contig sequence from the de novo assembly to generate male- and female-linked marker sequences.

Primer design and testing. We developed primers from the candidate male-linked and female-linked markers from our ddRAD-seq library using Primer-BLAST (Ye et al. 2012). We then tested each of the candidate markers on subsets of female gametophytes, male gametophytes, and tetrasporophytes from Mangoku-ura and Fort Johnson, including a negative control, in a 20 μL reaction volume that included: 2 μL of DNA template; 0.5 U GoTAQ Flexi-DNA Polymerase (Promega); 1x green reaction buffer; 250 μM of each dNTP; 1.5 mM of $MgCl_2$; and 200 nM each of the forward and reverse primers. Markers were tested using the following PCR cycling profile: one cycle of 10 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at 59°C, and 30 s at 72°C; and one cycle of 5 min at 72°C. All PCRs were performed using either a SimpliAmp or ProFlex thermocycler (Applied Biosystems, Foster City, CA, USA). PCR products were analyzed using 1.5% agarose gel electrophoresis stained with GelRed (Biotium, Fremont, CA, USA) and a 50-bp ladder (New England Biolabs, Ipswich, MA, USA) as size marker.

Next, we re-designed male-linked primers in order to yield a larger PCR product. The small PCR products (~70 bp) from male-linked and female-linked loci were the same size, and therefore, a duplex PCR would result in products that could not be easily differentiated during gel electrophoresis. Also, scoring two independent PCRs also increases the chances for misidentification of sex or ploidy. We used BLAST to compare all candidate sex-linked markers against the ongoing draft male (B.A. Flanagan, S.A. Krueger-Hadfield, C.J. Murren, C.C. Nice, A.E. Strand, E.E. Sotka, unpub. data) and female *Agarophyton vermiculophyllum* genomes (S.A. Krueger-Hadfield, unpub. data). Based on candidate markers with no match in the other sex, we chose a female-linked marker with existing primers for a ~ 70-bp product. We, then, expanded the target sequence to ~ 2 kb flanking male-linked loci and re-designed primers for a ~ 300-bp male-linked PCR product. We tested these PCRs using the same conditions as described above.

Sex-linked locus genotyping. In order to test the efficacy of the markers, we used thalli for which we had distinct information of reproductive status, geographic origin, and prior microsatellite data (Table 1). In total, we tested our markers on 20 female, 22 male, and 57 diploid thalli. In addition, we tested 35 vegetative thalli that were reproductive at the time of sampling (see Table 1 for the number of thalli from each of the sites sampled). Of the Fort Johnson samples (Lees et al. 2018), 22 were visually identified as vegetative and two as male at the time of sampling, seven of which had microsatellite data. Of the 2015 samples from Wachapreague, VA, all 16 had previously been genotyped with microsatellites (data from: Krueger-Hadfield et al. 2017, Bippus et al. 2018, Sotka et al. 2018). Using the candidate female- and male-linked markers, we amplified these thalli along with a female, male, tetrasporophyte, and negative controls in a 20 μL duplex PCR volume that included: 2 μL of DNA template; 0.5 U GoTAQ Flexi-DNA Polymerase (Promega, Madison, WI); 1x green reaction buffer; 250 μM of each dNTP; 1.5 mM of $MgCl_2$; 200 nM each of the male-linked forward and reverse primers; and 300 nM each of female-linked forward and reverse primers. The duplex PCR cycling profile was as follows: one cycle of 10 min at 95°C; 35 cycles of 30 s at 95°C, 30 s at 59°C, and 30 s at 72°C; and one cycle of 5 min at 72°C. PCR products were analyzed using 1.5% agarose gel electrophoresis stained with GelRed (Biotium, Fremont, CA, USA) and a 50-bp ladder (New England Biolabs, Ipswich, MA, USA) as size marker.

RESULTS

ddRAD-seq sex-linked marker candidates and primer design. The 20 male gametophytes had 9.9×10^6 mapped reads, the females had 5.3×10^6 mapped reads, and the tetrasporophytes had 2.0×10^7 mapped reads. When we filtered data stipulating at least 1 read per contig, we found 81,420 putative male-linked contigs and 25,056 putative female-linked contigs. We, then, increased the requirement to 400 reads per contig, yielding 36 male-linked and 19 female-linked contigs. Following further refinement, we used at least 500 reads per contig which enabled us to identify 19 candidate contigs present in males and tetrasporophytes, but not females, providing initial evidence suggesting they may be male-linked markers (Table S3 in the Supporting Information). We found seven candidate contigs present in females and tetrasporophytes, but not males, suggesting female-linked markers (Table S3 in the Supporting Information).

We were able to design primers in order to amplify a ~ 70-bp product in six of the seven female-linked markers and in 17 of the 19 male-linked markers (Table S3). For the female-linked markers, two (fem_o03 and fem_05) produced bands only in the females and tetrasporophytes, whereas the other four markers produced bands in

TABLE 2. Sex-linked locus information. Primer design: ddRAD-seq (primers designed from original ddRAD-seq contig), genomic (primers re-designed using the draft genome, male only [M] or male and female draft genomes [M + F]); Sex-linked: bioinformatically determined as a female- or male-linked marker; Scaffold: contig number from ddRAD-seq library (see sequence information in Table S3); Matches: written as male genome alignment matches, female genome alignment matches - 0 (only found in female or male g draft genome, respectively), 1 or greater (number of matches found across female and male draft genomes; for example, 1, 2: 1 match in male draft genome, 2 matches in female draft genome), - (not compared to the draft genomes); Profile: female (female-linked and only female/tetrasporophyte amplification), male (male-linked and only male/tetrasporophyte amplification), all (putative female- or male-linked marker but with amplification in both sexes), mix (some PCRs generated sex-specific patterns whereas other PCRs amplified in both sexes), none (no amplification). The loci in bold were ultimately determined to be the most reliable for sex and ploidy determination.

Locus	Primer design	Sex-linked	Scaffold	Primer sequence 5' to 3'	Expected size (bp)	Matches	Profile
fem_01	ddRAD-seq	Female	12905	F: TTGATGCTGATGCGTCTTTGG R: CCCATACCGAACAGGAGGATT	70	4.4	All
fem_02	ddRAD-seq	Female	1662	F: GCCCTGACGAAATGGATACG R: GGCACACGTTACACATGCTT	71	9.14	All
fem_o03	**ddRAD-seq**	**Female**	**3703**	**F: AGACTGGATTGCCGTCTGAG** R: TAGTTTCTCTCCATGTGCCAGC	**73**	**0.1**	**Female**
fem_04	ddRAD-seq	Female	4148	F: GGCTTCTCCCATGGATAATGCTG CAGAAGTTTTGCCACAGATCTTCAA	70	4.8	All
fem_05	ddRAD-seq	Female	6886	F: CGTAGCATGGGCCACTAGTTT R: GTCAAAAACGTCAACAGCCAGT	76	0.1	Female
fem_06	ddRAD-seq	Female	8996	F: GGGACGAGTCCGAGTCATAG R: GATATGGTGACCGTGCTCGC	75	1.3	All
mal_01	ddRAD-seq	Male	10882	F: TTCTTCTTTGGAGCCATGTGT R: TCGGTAGAGAAACATTCACGTC	72	1.0	Male
mal_n01	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: AGAAACGTCCCAACAGCACT R: TGAGCCCGAGGTGTTATGTG	244	–	All
mal_02	ddRAD-seq	Male	11733	F: CGCCGTATGACTTTTTCATGT R: TTGTCATCGGTAAGTCTGACAAT	74	1.0	Mix
mal_n02	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: CTTGAGTACTTCCCCTGCCG R: CGGTCGCACATGGAAGTTTG	248	–	All
mal_03	ddRAD-seq	Male	13325	F: GTCGTTTTTCAAGGGTAGCACT R: GTCTAGCACAAATGGAGCAATGA	80	1.0	Male
mal_n03	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: CATAGGCTCGATGTCCAGGG R: TGGGCAAAAGTTTGCGTGTT	277	–	Male
mal_04	ddRAD-seq	Male	1742	F: ACTTTCGCTCACCAGTTCCTAA R: GTTTAGTACCCCACGCATTTCTG	80	3,10	All
mal_05	ddRAD-seq	Male	2520	F: CAAACACTCAGCGAAGGGGG R: CCAGGTCGCATCATAGCAGTG	72	1,557	All
mal_06	ddRAD-seq	Male	4259	F: CGTAGATGGCGCTGAATAGC R: ACCCATCTGGGGTAAAGCTG	78	1,0	Male

(continued)

TABLE 2. (continued)

Locus	Primer design	Sex-linked	Scaffold	Primer sequence 5' to 3'	Expected size (bp)	Matches	Profile
mal_07	ddRAD-seq	Male	4434	F: TGTGCCTTCCTCCTTTATCGAA R: CTATGAGAAATTCTGCCGCCG	77	1,1	None
mal_08	ddRAD-seq	Male	4544	F: CTGCTTCCTTCAGCAATATCAATC R: AAAAGCGGCTCATAGCAAACTA	74	4,2	All
mal_09	**ddRAD-seq**	**Male**	**5889**	**F: CTCGTGACAGCGATTACCTGA** R: AACTCCGGTACGAGTCGTTG	**75**	**1,0**	**Male**
mal_n09	**Genomic – M + F**	**Male**	**Sc6Lk9p_379; HRSCAF = 462**	**F: CTCGTTCCGTAGGTGTGACC** R: GCCCCTGAACCACCTTGTAA	**270**	**–**	**Male**
mal_10	ddRAD-seq	Male	6386	F: GTTACGGAACGGCTCTAGACT R: ACGTGTGGCTAAGTGGATGA	80	1,2	All
mal_11	ddRAD-seq	Male	7132	F: TATGCTCGACATAAGTTCTGAGAGG R: ATGTCCGTGTAAGACCCGGC	70	1,1	All
mal_12	ddRAD-seq	Male	7505	F: GAAAACCTGCGAATATTGTCAGAAA R: GGCAAAGGAGCACTCAACCA	72	1,0	Male
mal_n12	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: CACTGTCGAGCCACTCTTGA R: AGCAGTACGATGCTCCACAC	241	–	Mix
mal_13	ddRAD-seq	Male	7532	F: TTCCGAACAACGTTCCTGAG R: CTGTACAGAGAGGTGTGAGATTC	75	1,0	Male
mal_n13	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F; CTGAGGACCAGCACGATGTC R: TCTTTCCGACACCACAACGA	213	–	All
mal_14	ddRAD-seq	Male	7787	F: CCTTGATGTGAGGCAGATTCCAT R: CGTGCATCCAGTTGCAGATCA	73	1,0	Male
mal_n14	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: TGCGGAGCATCTTGTCCATT R: TGAGAAAGGAGTCCACACGC	282	–	All
mal_15	ddRAD-seq	Male	7927	F: CGGAGTCGGAGAAAACATGCC R: AGGTGCTACCACATGTTCGGTC	70	1,5	None
mal_16	ddRAD-seq	Male	8582	F: ACGTGCTAGCTCCGTATTGG R: CTTTTTACTTCGATCCGCGCC	78	1,0	Mix
mal_n16	Genomic – M + F	Male	Sc6Lk9p_379; HRSCAF = 462	F: GAAGACGTACCCCGAGGTTG R: TTCATGTGACTACGCACCCC	246	-	All
mal_17	ddRAD-seq	Male	8755	F: TGGGTGAGAACTCCAGACCA R: ACAGCTGATCCAGAAAAACGAC	74	2,1	All

all three life cycle stages (Table 2). Six of the male-linked markers produced bands in the males and tetrasporophytes only, whereas six produced bands in all three life cycles stages, two did not amplify, and two produced variable amplification patterns in which one PCR would produce male and tetrasporophyte bands and then a subsequent PCR would produce bands in all three life cycle stages (Table 2).

Based on these results, we took advantage of the ongoing *Agarophyton vermiculophyllum* male and female draft genomes to search for matches in which our putative female-linked and male-linked contigs were found in both male and female genomes. The female-linked fem_o03 and fem_05 were only found in the female genome, whereas the other four putative female-linked contigs based on the RAD-seq filtering were found in both male and female genomes and amplified in all three life cycle stages (Table 2). Similar patterns were found for male-linked markers where markers that had male/tetrasporophyte banding patterns were only found in the male genome (Table 2). However, for two male markers (mal_02 and mal_16), though they were only found in the male genome, they produced variable PCR results with inconsistent amplification.

Using the nine male-linked markers identified from the analysis of matches from local alignments in both genomes, we re-designed male-linked primers to amplify a ~ 250-bp product (these markers are designated with an "n" before the number in Table 2). Only two male-linked markers amplified in male and tetrasporophyte thalli only (male_n03 and mal_n09; Table 2). Thus, due to more consistent amplification across Chelex-extracted DNA, we chose to use fem_o03 and mal_n09 as diagnostic markers for sex determination in *Agarophyton vermiculophllum*.

All of our sex-linked markers were located within a ~ 500 Mb region on one of the largest scaffolds of the draft male genome (see Table S3; B.A. Flanagan, S.A. Krueger-Hadfield, C.J. Murren, C.C. Nice, A.E. Strand, E.E. Sotka, unpub. data).

Determination of sex and ploidy in Fort Johnson, Mangoku-ura, and Wachapreague thalli. We tested amplification in the thalli used for the RAD-seq library (n = 20 females, n = 20 males, n = 16 diploids), and all females had the female band, all males had the male band, and all diploids had both the female and male bands.

The 16 thalli from Wachapreague had previously been genotyped with 10 microsatellites (Krueger-Hadfield et al. 2017). Eleven were determined to be tetrasporophytes and five as vegetative at collection. All 16 thalli displayed two bands using the sex-linked duplex PCR (Table 3) and previously had at least one, but as many as five, heterozygous microsatellite loci depending on the thallus, thus confirming that all were tetrasporophytes (Table S1b).

In addition to the Wachapreague thalli, we scored eight thalli that were previously genotyped using the microsatellites (Krueger-Hadfield et al. 2017) and three thalli that were scored for reproductive structures, but not genotyped from field collections at Fort Johnson in 2015. One vegetative thallus was determined to be a female gametophyte (FJS_063; Fig. 2b, Table 2). Nine thalli (N = 5 visually identified as tetrasporophytes, N = 4 as vegetative) were determined to be tetrasporophytes (Table 2). For the genotyped tetrasporophytes, the number of heterozygous microsatellite loci ranged from 2 to 7. However, there was a single thallus (FJS_091) visually identified at the time of sampling as a tetrasporophyte that was subsequently determined to be haploid based on the microsatellite multilocus

TABLE 3. The comparison between morphological and molecular determination of life cycle stage from thalli sampled from PVC recruits at Fort Johnson (see also Lees et al. 2018) and at Fort Johnson and Wachapreague as part of an invasion history study (Krueger-Hadfield et al. 2017). Morphological determination was based on visual determination of reproductive structures at 40x. Molecular determination was based on the duplex PCR of fem_o03 and mal_n09 sex-linked markers. None of the PVC recruits have been genotyped with microsatellites (Kollars et al. 2015, Krueger-Hadfield et al. 2016). However, we did include 8 thalli from Fort Johnson and 16 from Wachapreague that had been previously genotyped at 10 microsatellites by Krueger-Hadfield et al. (2017) and are provided in Table S2b.

Site	Ploidy	Sex	Morphological determination	Molecular determination
Fort Johnson – PVC recruits	Tetrasporophyte	–	0	6
	Gametophyte	Female	0	10
	Gametophyte	Male	2	8
	Vegetative	–	22	–
Fort Johnson – 2015 invasion history sampling	Tetrasporophyte	–	6[a]	9
	Gametophyte	Female	0	1
	Gametophyte	Male	0	1[a]
	Vegetative	–	5	–
Wachapreague – 2015 invasion history sampling	Tetrasporophyte	–	11	16
	Gametophyte	Female	0	0
	Gametophyte	Male	0	0
	Vegetative	–	5	–

[a]One thallus was considered a tetrasporophyte based on morphological determination, but based on the microsatellites and sex-linked markers, was found to be a male gametophyte.

genotype (Krueger-Hadfield et al. 2017). Using our PCR assay, we confirmed that this thallus was indeed a haploid, male gametophyte (Fig. 2b, Table 2).

The 24 thalli sampled from the PVC settlement posts were identified as male gametophytes ($N = 2$) and vegetative ($N = 22$) at the time of sampling. The duplex PCR described above using fem_o03 and mal_n09 was amplified and scored blindly without morphological or microsatellite confirmation. Six thalli were considered as tetrasporophytes due to the presence of two bands, 10 as female due to the presence of the female band, and eight as male due to the presence of the male band (Fig. 2c, Table 3). The two morphologically identified male gametophytes were also confirmed as male based on the PCR assay (Fig. 2c).

DISCUSSION

We used ddRAD-seq to identify female- and male-specific markers in the widespread red macroalga *Agarophyton vermiculophyllum* and validated the sex specificity of these markers using PCR. As previously reported by Gamble and Zarkower (2014) and Fowler and Buonaccorsi (2016) in other taxa, we demonstrate that RAD-seq can be a powerful method with which to rapidly characterize sex-linked markers in nonmodel organisms that lack genomic resources. We discuss a workflow that can be used by other psychologists, the utility of these markers for understanding sex chromosome evolution in red algae, and the information these markers can provide for studies for further elucidating the *A. vermiculophyllum* invasion.

At the outset of our study, there were no draft genomes for *Agarophyton vermiculophyllum*, or other species within the Gracilariales. None of our microsatellites appeared to be sex-linked (Kollars et al. 2015, Krueger-Hadfield et al. 2016) as had been found in other kelps (e.g., Shan and Pang, 2010). While we only tested 66 RAPDs, we did not find a single marker that was reliably sex-specific (see Appendix S1), which has been successful in other species. Guillemin et al. (2012), as an example, found four sex-linked RAPD markers out of 180 tested, and based on a review of other studies, determined that their success rate was quite high. Nevertheless, some studies tested hundreds of RAPDs to no avail (e.g., McLetchie and Tuskan 1994, Polley et al. 1997). Rather than continuing to test RAPDs, we took advantage of an available ddRAD-seq library (B.A. Flanagan, S.A. Krueger-Hadfield, C.J. Murren, C.C. Nice, A.E. Strand, E.E. Sotka, unpub. data) developed as part of our ongoing study of the invasion history of *A. vermiculophyllum* (Krueger-Hadfield et al. 2016, 2017, Sotka et al. 2018).

While genomic resources are rapidly becoming available for macroalgae (Cock et al. 2010, Collén et al. 2013, Brawley et al. 2017), RAD-seq approaches outlined here would allow for psychologists interested in breeding programs, population genetics and demography, and general eco-evolutionary questions to develop a diagnostic tool without an available genome (see generalized workflow in Fig. 3). The first three parts of the workflow, in which reproductively mature female gametophytes, male gametophytes, and sporophytes are sequenced using RAD-seq, are the same steps whether a genome is available or not. RAD-seq data are demultiplexed, and putative female- or male-linked sequences are separated from reads that are found in both males and females. Sex-specific reads are cross-checked such that female-linked sequences are only found in the female and sporophytic libraries and vice versa for the male-linked sequences. Primers can then be easily designed for the short sequences generated by our library approach and almost all amplified PCR products of 70-80 bp in size. These products were easily distinguished from any primer dimer with 1.5% agarose gels (Figs. 2, 3d). We note that future efforts in species that lack an assembly would likely benefit from longer reads (e.g., 200 bp) than the relatively shorter reads used here (~100 bp) as these may allow more flexibility in designing a duplex PCR assay, particularly without a genome.

If a taxon of interest has no genomic resources, assays can still proceed (shown in Fig. 3d). Female- and male-linked sequences are roughly the same size precluding duplexing, unless longer reads are generated in the RAD-seq libraries. Restriction fragment length polymorphism (RFLP) analyses, such as those performed by Fowler and Buonaccorsi (2016) to distinguish sexes, would be difficult on ~ 100-bp fragments. However, separate PCRs and gels can be run for the female- and male-linked markers. Females should have a band in the female marker and not in the male, vice versa for males, and sporophytes should have bands in both assays. The downside to this assay is that scoring is, in part, relying on the absence of a band. Individual PCRs could fail such that a sporophyte is scored as either a female or a male. This is a problem experienced by others working in heterogametic sex chromosomal systems, such as Gamble and Zarkower (2014), in which markers linked to the heterogametic sex amplify, but there is no band present in the homogametic sex. In the case of Gamble and Zarkower (2014), the authors included an autosomal gene as an internal positive control. We recommend including a similar positive control to ensure successful PCR, but we did not explore this further as we had access to other genomic resources in *Agarophyton vermiculophyllum* with which to design a larger male PCR product and duplex PCR assay.

If a draft or annotated genome is available, as in our case, then researchers can cross-check their sex-linked markers against an available male or female genome (Fig. 3e). This will enable the characterization of larger PCR products that in turn facilitate

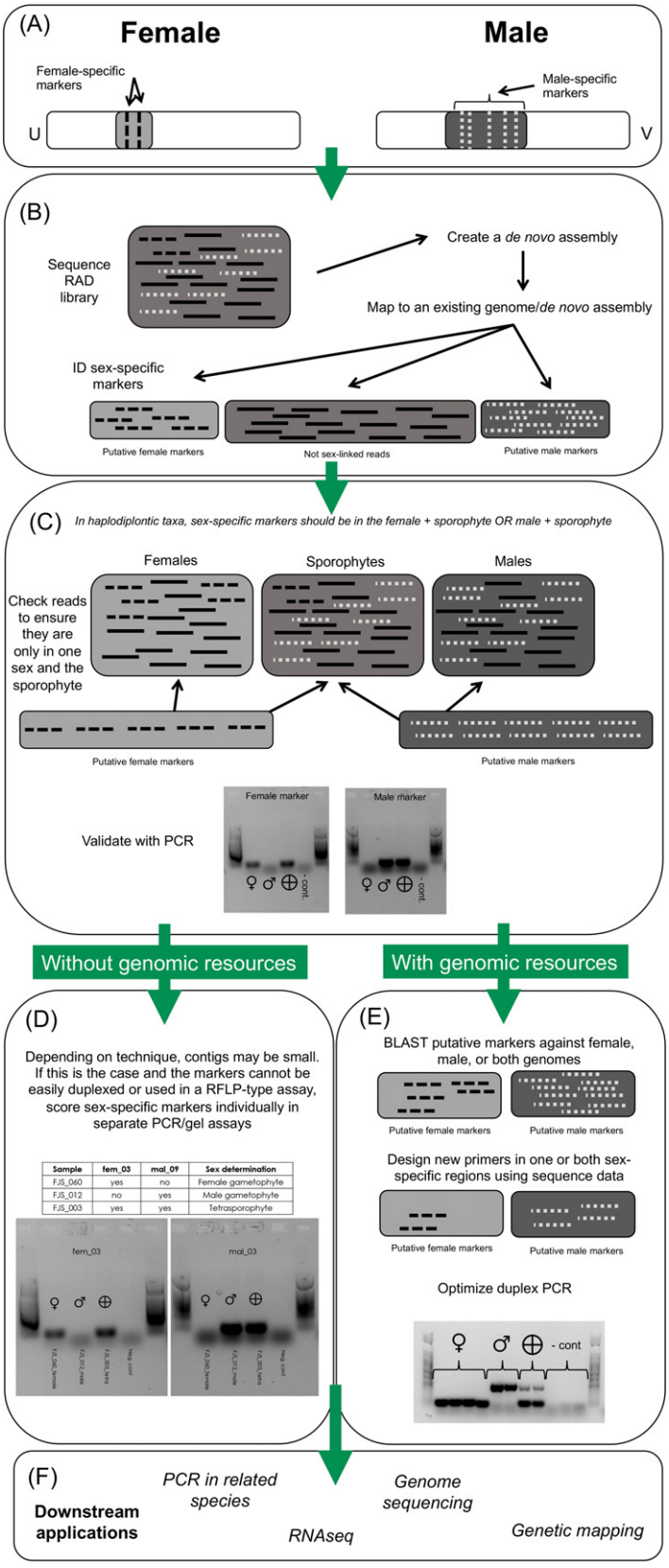
(A)
Female
Male
Female-specific markers
Male-specific markers
U
V
(B)
Sequence RAD library
Create a de novo assembly
Map to an existing genome/de novo assembly
ID sex-specific markers
Putative female markers
Not sex-linked reads
Putative male markers
(C)
In haplodiplontic taxa, sex-specific markers should be in the female + sporophyte OR male + sporophyte
Females
Sporophytes
Males
Check reads to ensure they are only in one sex and the sporophyte
Putative female markers
Putative male markers
Validate with PCR
Female marker
Male marker
- cont.
Without genomic resources
With genomic resources
(D)
Depending on technique, contigs may be small. If this is the case and the markers cannot be easily duplexed or used in a RFLP-type assay, score sex-specific markers individually in separate PCR/gel assays
Sample | fem_03 | mal_09 | Sex determination
FJS_060 | yes | no | Female gametophyte
FJS_012 | no | yes | Male gametophyte
FJS_003 | yes | yes | Tetrasporophyte
fem_03
mal_03
(E)
BLAST putative markers against female, male, or both genomes
Putative female markers
Putative male markers
Design new primers in one or both sex-specific regions using sequence data
Putative female markers
Putative male markers
Optimize duplex PCR
- cont
(F)
Downstream applications
PCR in related species
RNAseq
Genome sequencing
Genetic mapping

FIG 3. Outline of the RAD-seq workflow for haplodiplontic algae. (a) A cartoon of female- (black dashed lines) and male-specific restriction sites (gray dotted lines) located in the putative sex-determining region (gray region) on the U and V chromosomes, respectively. The solid black lines refer to restriction sites that are shared between female and male gametophytes (i.e., not sex-linked). (b) Summary of the bioinformatic analysis of the RAD-seq libraries where raw reads are mapped to either a de novo assembly in the absence of a genome or to a genome. Putative female- (black dashed lines) and male-linked sequences (gray dotted lines) as well as nonsex-linked sequences (solid black lines) can be identified based on filtering parameters. Some of these putative sex-specific markers may be false positives at this stage. (c) Putative female- and male-linked markers need to be cross-checked to determine whether they are found in female and sporophyte reads or male and sporophyte reads, respectively. Reads are shown grouped as females, sporophytes, and males for simplicity. Markers that pass this filtering step can then be validated using PCR. (d) Without genomic resources (i.e., without a genome), assays can proceed with PCR products that are roughly the same size in two separate reactions and gels and scored sequentially. However, if deciding on a RAD-seq protocol, choosing larger fragments will enable PCR duplexing with two different sized products for male- and female-linked markers. However, without genomic resources, it will be challenging to cross-validate the efficacy and sex specificity of the markers beyond robust PCR testing. (e) With genomic resources (i.e., with a genome), it is possible to BLAST putative sex-linked sequences against a female genome, a male genome, or both to proceed with markers that have no matches and to design large PCR products for one or both of the sex-linked markers in order to duplex PCRs. (f) With either technique, though easier if there are other genomic resources, subsequent studies can embark PCR tests in related species, RNAseq, genome sequencing, genetic mapping, etc. in order to characterize the SDR. Adapted from Gamble and Zarkower (2014). [Color figure can be viewed at wileyonlinelibrary.com]

the development of duplex PCR. Duplexing PCRs decrease the chance of scoring errors due to PCR failure as the female- and male-linked markers are amplified in the same reaction. In this study, there were no BLAST matches against the male genome for the two promising, female-linked sequences (fem_o03 and fem_05; Table 2), whereas the other four putative female-linked sequences were found in the male genome. These latter four putative female-linked markers amplified in all three life cycle stages, suggesting that they are not female-linked. It is possible that restriction site differences, sequencing quality, or filtering issues may explain the lack of these four sequences in the male gametophytes sequenced as part of our library (see discussion in Gamble 2016). Gamble (2016) suggested that amplification in both female and male samples in a lizard was likely due to sex-specific restriction sites that were flanked by conserved sequence in both sexes. This could be the case in *Agarophyton vermiculophyllum* as well, but ongoing sequencing efforts to characterize the red algal sex-determining region (SDR) will likely shed light on those patterns. Nevertheless, we were still able to identify several female and male-linked markers without the male or the female genome. Our genomic resources aided in marker characterization and allowed for further understanding of cross-amplification in putative sex-linked sequences, but were not necessary to develop an efficient sex-linked marker assay.

For other macroalgal taxa, similar RAD-seq methods as shown in Figure 3 can be relatively easily undertaken. With 20 thalli from two sites, we found a handful of candidate markers, but as Gamble and Zarkower (2014) demonstrated through re-analyses of subsets of their data, small sample sizes can lead to false positives and require increased validation steps via PCR. For red algae, males can be difficult to find as they may be more ephemeral, have more narrow windows of maturity, or be harder to determine in the field (Tveter-Gallagher et al. 1980, Krueger-Hadfield et al. 2015). However, sample sizes of female gametophytes, male gametophytes, and sporophytes should not be as challenging to obtain as collecting and identifying samples for some vertebrates that may be hard to properly sex or are endangered, leading to small sample sizes (Gamble and Zarkower 2014, Gamble et al. 2015).

Upon validating one female- and one male-linked marker and developing the duplex PCR, we found that Chelex-extracted DNA amplified well. Chelex is inexpensive compared to spin columns, such as the Macherey-Nagel Nucleospin® kit we used for library preparation. An additional benefit of Chelex extractions is that they are much faster as they take approximately an hour, including sample preparation, as compared to several hours for a spin column kit. Thus, it would be possible to extract 192 thalli using racks of tube strips (e.g., Macherey-Nagel ref 740477) and perform PCR (~70 min) and gel electrophoresis in a single day. While reproductive thalli need not necessarily be screened, we misidentified a thallus as a tetrasporophyte based on visual observations of reproductive structures. Based on a combination of our new sex-linked marker and microsatellite genotyping, this thallus was confirmed as a male gametophyte (Table 3, Fig. 2b). It is possible that this thallus was in the process of becoming reproductive, and immature spermatangial sori were mistaken for empty tetrasporangial sori. Thus, screening all thalli with a sex-linked marker could cross-check field data, especially for taxa that require microscopy (e.g., a dissecting microscope) in order to determine reproductive state, such as male gametophytes or tetrasporophytes in *Agarophyton vermiculophyllum*. Chelex extractions, importantly, do not require a lot of tissue. We used ~ 1 cm of dried thallus tissue, leaving plenty of tissue for subsequent extractions for downstream analyses that may require a large amount of high molecular weight DNA. This is especially relevant for red algal female gametophytic tissue. The carposporophyte contains male DNA in the developing carpospores and can readily be

detected when genotyping the cystocarps (Engel et al. 1999, Krueger-Hadfield et al. 2015) or when accidentally including cystocarps and using polymorphic markers, such as microsatellites (S.A. Krueger-Hadfield, unpub. data). For terete thalli, such as found in *A. vermiculophyllum* (see Fig. 1), we recommend removing cystocarps prior to preservation in silica gel as cystocarps cannot be easily seen or removed following desiccation. Removing cystocarps is a laborious step after sample collection and may yield only a small amount of suitable female tissue for subsequent DNA extraction.

Routine screening of thalli would also shed light on intraspecific life-history variability, a common phenomenon in many red algae (Maggs 1988). Kain and Destombe (1995) reviewed the life-history variation in the Gracilariales and discussed aberrant reproductive structures in which male and/or female reproductive organs occur on the same thallus with or without tetrasporangia. Sex-linked markers may be useful diagnostic tools to explore abnormal life-history patterns quickly and inexpensively. Moreover, in isomorphic taxa, sex-linked markers would facilitate downstream population genetic data collection and interpretation. If thalli are vegetative at the time of sampling, microsatellites would take more time and be more costly in order to determine ploidy (see also Krueger-Hadfield and Hoban 2016). Importantly, sex-linked marker PCR assays would characterize the female and male components of populations. For macroalgae, studies have always relied on reproductive structures for male and females, even when chemical tests are capable of distinguishing between gametophytes from sporophytes (Lazo et al. 1989, Scrosati and DeWreede 1999, Thornber and Gaines 2004). Ellis et al. (2017) have recently demonstrated the importance of distinguishing among sexes as sex can have significant physiological, biochemical, and reproductive consequences that ultimately affect survival. In their review, the authors found fewer than 4% of the studies investigated sex differences in response to ocean acidification, but when sexes were distinguished, there were significant differences in the responses of females and males, suggesting that overlooking sex may be obscuring important responses to abiotic and biotic factors. For many macroalgal ecophysiological studies, ploidy and/or sex are not always considered (but see Oppliger et al. 2012 as an example). For example, Cornwall et al. (2020) compared the responses of juvenile crustose coralline algae to ocean acidification, but did not determine ploidy or sex.

In the case of *Agarophyton vermiculophyllum*, these markers represent a major step forward for the exploration of the evolutionary ecology of this widespread invasion. In previous work, many thalli throughout the Northern Hemisphere, particularly in non-native, free-floating habitats, are vegetative (Krueger-Hadfield et al., 2016, 2017, 2018). Our sex-linked marker will allow for rapid assays of populations. At a subset of free-floating non-native populations, some vegetative thalli were considered haploid because they were fixed homozygotes at our 10 microsatellite loci (Krueger-Hadfield et al. 2016). Previous population genetic work in *Chondrus crispus* demonstrated that a small percentage of genotyped tetrasporophytes, determined based on a combination of reproductive structures and the resorcinol test, were fixed homozygotes (Krueger-Hadfield et al. 2013). It is possible that some fixed homozygotes in our free-floating *A. vermiculophyllum* populations are tetrasporophytes with very low heterozygosity. For example, some tetrasporophytes (confirmed by visual observation, the 10 microsatellite loci, and the sex-linked markers from this study) from Wachapreague only had a one heterozygous microsatellite locus, suggesting the possibility of low heterozygosity across our 10 loci. With these tools in hand, we can screen thalli using these markers and begin to more accurately explore mating system and demographic variation across the current range of this macroalga.

In summary, we have developed DNA-based sex markers for the widespread invader *Agarophyton vermiculophyllum* using a workflow that should be readily adaptable for other taxa with or without genomic resources. This work will contribute to our understanding of sex determination in algae building on the pioneering work in brown algae (Cock et al. 2010, Ahmed et al. 2014, Lipinska et al. 2015, Coelho et al. 2018). Not only will these markers greatly aid ongoing studies of the role of evolution during the invasion of *A. vermiculophyllum,* but also the development of sex-linked markers in other taxa will greatly expand our understanding of the evolutionary ecology of haplodiplontic algae in general.

We thank G. Bonthond, M.L. Guillemin, J.M. Cock, and S. Coelho for helpful discussions; and C.R. Hadfield, J. Pocklington, H. Endo, S.J. Shainker, A. Gaffney, and S. Fate for help in the field. We also thank M.C. Oliveira and the anonymous reviewers for their comments that improved the manuscript. This project was supported by start-up funds from the University of Alabama at Birmingham and NSF BIO-OCE (1357386).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. UBC RAPD primers and amplification success with 35 or 45 cycles.

Table S2. (A) Microsatellite genotypes for the haploid gametophytes, previously published in Krueger-Hadfield et al. (2017) and Lees et al. (2018) in GenAlEx format. (B) Microsatellite genotypes for the diploid tetrasporophytes, previously published in Krueger-Hadfield et al. (2017) and Lees et al. (2018) in GenAlEx format.

Table S3. Female- and male-linked sequence information. Marker name; whether a primer was designed; the marker development type (RAD-seq or genome); the scaffold or contig where the marker was found; the mean number of reads; the standard deviation; the median number of reads; the maximum number of reads; the proportion of individuals that had reads; the sequence.

Appendix S1. RAPD Screening.

Appendix S2. Sex-specific contigs from RAD-Seq data.