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ABSTRACT
Haploid-diploid life cycles impose unique eco-evolutionary consequences, rendering commonly used proxies difficult to use (e.g. separate sexes prevent selfing). Population genetic analyses are therefore required to explore patterns of reproductive system variation. However, there are still few haploid-diploid species for which polymorphic, nuclear loci exist. This problem is particularly acute for algae. Here, we describe the development of the first microsatellite loci in a freshwater red alga. We tested 73 candidate loci against a panel of *Batrachospermum gelatinosum* (L.) De Candolle gametophytes that encompass much of its North American range. Ten loci consistently amplified and were characterized by clean peak architectures on a capillary sequencer with one allele per locus, as expected in a haploid gametophyte. We then explored some basic population genetic indices in gametophytes collected from one site and obtained good resolution based on the probability of identity (*pid*). Yet, we observed a pattern of clumped repeated genotypes throughout the stream reach sampled. The pattern of moderate genotypic richness could be due to intragametophytic selfing resulting in the complete loss of genetic diversity from a single gamete union. Future studies will need to sample more populations to determine if intragametophytic selfing is the dominant reproductive mode in this monoicous taxon. The loci developed here represent an important tool for studying freshwater red algal populations in specific as well as enhancing our understanding of reproductive system variation and the haploid-diploid life cycle of algae in general.

KEY WORDS
North America, Rhodophyta, haploid-diploid life cycle, locus development, partial clonality, primers, population genetics.

RÉSUMÉ

Développement des microsatellites chez l'algue rouge d'eau douce Batrachospermum gelatinosum *(L.) De Candolle (Batrachospermales, Rhodophyta).*

L'étude des conséquences éco-évolutionnaires des cycles haplo-diplophasiques sur les variations du système de reproduction par le biais de la génétique des populations nécessite le développement de marqueurs génétiques adaptés. Cependant, peu de marqueurs polymorphiques nucléaires sont disponibles pour les espèces haplo-diploïdes, notamment les algues. Dans cette étude, nous décrivons le développement des premiers marqueurs microsatellites chez une algue rouge d'eau douce. Nous avons testé 73 locus candidats sur un ensemble de gamétophytes de *Batrachospermum gelatinosum* (L.) De Candolle représentatifs des populations d'Amérique du Nord. Parmi les locus testés, dix ont été amplifiés par PCR avec succès et présentent une architecture de pics lisibles sur séquenceur capillaire avec un seul allèle comme attendu lors du génotypage de gamétophytes haploïdes. Nous avons ensuite utilisé ces marqueurs pour réaliser des analyses basiques de génétique des populations sur des individus échantillonnés dans un site en Alabama, ce qui a révélé une bonne résolution des marqueurs basée sur la probabilité d'identité (*pid*). Pourtant, nous avons trouvé des groupes de génotypes répétés spatialement proches dans le site échantillonné. Ce patron de richesse génotypique modéré pourrait être dû à des croisements consanguins entre gamètes issus du même gamétophyte qui résulteraient en la perte totale de diversité génétique. Les futures études devront inclure un échantillonnage plus large pour voir si l'autofécondation est le mode de reproduction dominant chez cette espèce monoïque. Les outils génétiques développés dans cette étude nous permettent de mieux comprendre des populations d'algues rouges d'eau douce ainsi que les variations du système de reproduction lié au cycle biphasique des algues.

MOTS CLÉS
Amérique du Nord,
Rhodophyta,
cycle de vie haploïde-diploïde,
développement des locus,
clonalité partielle,
amorces,
génétique des populations.

INTRODUCTION

The reproductive system describes the relative rates of sexual and asexual reproduction in a population (Barrett 2011). It is the key life history trait that varies widely among organisms (Barrett 2014), influencing the partitioning of genetic diversity within and among populations (Hamrick & Godt 1996) and the maintenance of genetic associations (Otto & Marks 1996). Outcrossing typically results in genetically diverse populations, whereas self-fertilization (or selfing), inbreeding, and asexuality reduce genetic diversity and effective recombination rates. Otto & Marks (1996) suggested selfing, inbreeding, and asexuality should lead to an increase in the duration of the haploid stage, and thus, a correlation between the reproductive system and the life cycle. However, tests of this correlation remain rare, largely because most available data on reproductive mode variation are from ecologically diploid angiosperms (Whitehead *et al.* 2018).

Algae have great potential for understanding the relationship between the reproductive mode and the life cycle. Both micro- and macroalgal taxa exhibit tremendous variation in life cycle types and reproductive systems. However, the haploid-diploid life cycle, in which multicellular gametophytes and sporophytes alternate, generates unique consequences that challenge traditional understanding and the utility of common proxies used to describe patterns in nature (Krueger-Hadfield 2020; Stoeckel *et al.* 2021a). For example, many algae are partially clonal simultaneously undergoing sexual (i.e., selfing to outcrossing) and asexual reproduction. Asexual reproduction varies tremendously from fragmentation (e.g. *Gracilaria* spp.; Kain & Destombe 1995) to asexual spore production (see Maggs 1988). While the balance between sexual and asexual reproduction strongly influences ecological (e.g. Halkett *et al.* 2005) and evolutionary success (e.g. Orive *et al.* 2017), the eco-evolutionary consequences of partial clonality remain largely uncharacterized because population genetic models have been developed from exclusively sexual or asexual species (Stoeckel *et al.* 2021a, b). Moreover, while in angiosperms, separate sexes are often used as a proxy for outcrossing as selfing cannot occur, this is not the case in haploid-diploid taxa. Separate sexes (or dioicy since sex is determined at the haploid gametophyte stage; Beukeboom & Perrin 2014) do not prevent selfing (i.e., intergametophytic selfing; Klekowski 1969) when the male and female gametophytic pair share the same sporophytic parent. Moreover, in hermaphroditic (or monoicous) gametophytes, one event of selfing generates instantaneous, genome-wide homozygosity in the sporophytic offspring (Klekowski 1969). The ratio of hermaphroditism to separate sexes among algal lineages varies tremendously (Bringloe *et al.* 2020), suggesting a comparable, yet distinctive axis of variation from selfing to outcrossing as compared to angiosperms (Olsen *et al.* 2020). Thus, we cannot resolve some of these patterns in nature without population genetic data (Tibayrenc & Ayala 1991; Ellegren & Galtier 2016).

Recently, Krueger-Hadfield *et al.* (2021) reviewed available studies for which both gametophytes and sporophytes had been genotyped using polymorphic markers and found only a handful of red algae with such data. To the best of our knowledge, only marine red algae have been explored from a population genetic perspective using co-dominant, polymorphic markers. The red macroalgae found in freshwater ecosystems have been overlooked. These red algae are nested within the marine reds, suggesting not only the invasion of freshwater ecosystems,

TABLE 1. — Sites in which *Batrachospermum gelatinosum* (L.) De Candolle gametophytes were sampled. The gametophytes were used in: **library**, SSR-enriched library preparation; **screen**, initial screening on agarose gels or the capillary sequencer; **popgen**, initial population genetic analyses to test locus efficacy. The sample size (**N**) is provided for each site.

Site name	Site abbreviation	State/ Province	Coordinates	Date	Collectors	Development	N
Yellow Creek	AL-YEC	Alabama	33°34'19.2"N, 87°24'10.8"W	2.V.2022	SJSC, APO, BMT	screen	1
Cripple Creek	AL-CRC	Alabama	33°29'33.108"N, 87°33'45.478"W	2.V.2022	SJSC, APO, BMT,	screen, popgen	28
Houston Branch	MD-HOU	Maryland	38°44'14.0"N, 75°44'52.4"W	19.VI.2022	RMC, MLV	screen	1
Conneaut Outlet	PA-COT	Pennsylvania	41°34'29.3"N, 80°13'07.6"W	30.IV.2022	RMC, MLV	library	1
Fuller Brook	CT-FLB	Connecticut	41°47'53.6"N, 72°04'08.6"W	12.IV.2022	RMC, MLV, CWS	screen	1
Chipuxet River	RI-CPR	Rhode Island	41°28'57.0"N, 71°33'04.0"W	11.IV.2022	RMC, MLV	library	1
Knappens Creek (Houghton Lake)	MI-HLK	Michigan	44°17'54.4"N, 84°38'57.6"W	12.V.2022	RMC, GAL, MLV	screen	1
Traverse River (Mohawk Gay Road)	MI-TRM	Michigan	47°15'45.3"N, 88°14'13.6"W	11.V.2022	SJSC, APO, BMT, SAKH	screen	1
Margaree River	NS-MAR	Nova Scotia	46°19'10.1"N, 61°02'23.7"W	24.VII.2022	MLV, WBC	screen	1

but also the subsequent loss of separate sexes with many species being monoicous (see Krueger-Hadfield *et al.* 2024). Moreover, freshwater red macroalgae have unique, haploid-diploid life cycles in which the macroscopic gametophyte is physically connected to the microscopic sporophyte (called the chantransia) (Sheath 1984). Krueger-Hadfield *et al.* (2024) highlighted the promise of these taxa, with an emphasis on the order Batrachospermales, to expand our understanding of reproductive system variation across the eukaryotic tree of life.

Here, we describe the development of polymorphic microsatellite loci with which to genotype the freshwater red alga *Batrachospermum gelatinosum* (L.) De Candolle (Fig. 1). This species is distributed throughout the Northern Hemisphere (Entwisle *et al.* 2009) and is likely the most common freshwater red alga in North America (Sheath & Cole 1992). Its wide distribution may be attributed to its ability to tolerate a wide range of chemical and physical stream characteristics (Vis *et al.* 1996). There is also phenological and morphological variation within and among populations (Vis *et al.* 1996; Vis & Sheath 1997; Drerup & Vis 2014). House *et al.* (2010) found little genetic variation throughout the geographic range of *B. gelatinosum* based on the mitochondrial *cox*1 and plastid *rbc*L genes. Thus, further studies are needed to integrate the link between stream characteristics and *B. gelatinosum* reproduction and gene flow. The markers we have developed are suitable for studies of reproductive system variation and patterns of gene flow in this species. Based on cross-amplification of other microsatellites across taxa (e.g. kelp; Coelho *et al.* 2014), these loci may also be useful for other *Batrachospermum* species. Nevertheless, they expand the available genetic resources for algae that should facilitate future eco-evolutionary studies.

MATERIAL AND METHODS

SAMPLE COLLECTION

We used several different sets of *Batrachospermum gelatinosum* gametophytes for the various stages of microsatellite development and testing. For single sequence repeat (SSR)-enriched genomic library construction (see more below), we used gametophytes from Conneaut Outlet, PA and Chipuxet River, RI (Table 1). We, then, used gametophytes collected from seven sites across *c.* 13 degrees of latitude encompassing much of the *B. gelatinosum* range in North America (Table 1) to test locus amplification (see more below). Finally, we collected 28 gametophytes at Cripple Creek, Tuscaloosa County, Alabama, United States for initial population genetic analyses using the newly developed loci (Table 1). At Cripple Creek, we haphazardly sampled gametophytes along a reach. We observed each gametophyte under the microscope for the presence of carposporophytes and to ensure that gametophytes were physically separated if entangled with one another. We removed the lower portion of the gametophyte if there was visible sediment to ensure we had a single gametophyte, and the chantransia and other detritus from the biofilm was removed. We preserved each gametophyte in silica gel, and when possible, remaining tissue was pressed to create herbarium vouchers that are housed at the Bartley Herbarium, Ohio University (BHO).

DNA EXTRACTION

We extracted total genomic DNA using the Machery-Nagel Nucleospin® Plant II kit (Macherey-Nagel, Cat #740663.24) following the manufacturer's methods, except for the cell lysis step in which we incubated the lysate at room temperature for one hour and then we eluted DNA in either 200 µL (for seven gametophytes for initial locus screening) or 100 µL of molecular grade water (28 gametophytes for population genetic analyses; see Krueger-Hadfield *et al.* 2013).

MICROSATELLITE LOCUS IDENTIFICATION

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 implemented in Ryan *et al.* (2021) and Heiser *et al.* (2023). 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 was 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 381 sequences with eight or more repeats, 192 of those had primers assigned, and 55 were potentially duplicated in the library. For trinucleotides, we identified 651 sequences with eight or more repeats, 263 of those had primers assigned, and 46 were potentially duplicated in the library. For tetranucleotides, we identified 195 sequences with six or more repeats, 59 of those had primers assigned, and six were potentially duplicated in the library. We had 137, 217, and 53 potential loci with di-, tri-, and tetranucleotide repeat motifs.

We used the R code provided by Schoebel *et al.* (2013) in R version 4.2.1 (R Core Team 2022) to combine the primer and microsatellite sequences into one file. For the dinucleotides, after merging the files we had 147 unique reads remaining. After removing duplicated forward and reverse primer sequences, we had 127 unique reads remaining. For trinucleotides, after merging the files we had 224 unique reads remaining. After removing duplicated forward and reverse primer sequences, we had 212 unique reads remaining. For tetranucleotides, after merging the files we had 54 unique reads remaining. After removing duplicated forward and reverse primer sequences, we had 52 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 from smallest (0°C) to largest (2.51°C). We filtered out loci with a temperature difference of greater than 1°C. We, then, filtered the putative loci by the forward penalty score, reverse penalty score, and the pair penalty score. In each category, we removed loci with a penalty score > 0.5. We chose the top 162 loci in which at least one of these four categories was fulfilled. Of these 162 loci, 94 fulfilled all four categories and we used in a BLAST search in Geneious Prime v.2022.2.2 (Biomatters, Ltd., Auckland, New Zealand; https://www.geneious.com) 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 73 candidate loci were chosen following the BLAST search and screened using seven gametophytes (see Table 1).

Microsatellite locus screening and PCR conditions

Candidate loci were amplified using simplex PCRs with a final volume 20 µL: 2 µL of neat DNA template, 250 nM of each forward and reverse primers, 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 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 two minutes, followed by 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 30 seconds, with a final elongation at 72°C for five minutes. Approximately 5 µL of each PCR product was screened on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, United States, Cat #41002-1). Each locus was then categorized based on the amplification profile: one band, multiple bands, or no amplification (Table 2). We considered primers to be good candidates if they amplified well across the seven gametophytes and only had one band per gametophyte. Based on these criteria, 18 candidate loci were selected for screening using the capillary sequencer.

We assigned dyes – 6FAM, NED, VIC, PET – to each forward oligo for the 18 candidate loci (Table 2). To plan for future multiplexing, we assigned dyes such that multiplexes will contain loci with different fragment lengths that can be easily distinguished from one another. We performed fragment analysis of all samples at the Heflin Center for Genomic Sciences at the University of Alabama at Birmingham. We diluted 1.5 µL PCR product in 9.7 µL HiDi formamide (Applied Biosystems) and 0.30 µL GS 500 LIZ (Applied Biosystems, Cat #4322682). We scored alleles using GENEIOUS PRIME. Loci were categorized based on their allelic profiles as one allele (expected as gametophytes are haploid) or multi-allelic (two or more alleles). We discarded multi-allelic loci and moved forward with loci that exhibited one allele per locus and were therefore considered to be in single locus genetic determinism (see Krueger-Hadfield *et al.* 2011).

Microsatellite allele binning

We used TANDEM to bin alleles while reducing rounding error (Matschiner & Salzburger 2009). We manually checked allele bins.

Data analyses

Gametophytes that had more than three loci with no amplification after multiple attempts at PCR were excluded from subsequent analyses. The frequency of null alleles was directly estimated for the remaining gametophytes for which there was no PCR product after discounting any technical errors (see also Krueger-Hadfield *et al.* 2011). As a preliminary exploration of these loci, we calculated the following summary statistics to describe the population of *B. gelatinosum* at Cripple Creek following the calculations provided in Krueger-Hadfield *et al.* (2021) and Stoeckel *et al.* (2021a): 1) the probability of identity between sibs (*pid*) to assess whether loci are of high enough resolution to distinguish among individuals; 2) genotypic richness (R) and evenness (D^*), which provide information on the relative proportion of unique multilocus genotypes (MLGs) and their distribution, respectively; 3) linkage disequilibrium ($\bar{r}_D$) following Agapow & Burt (2001); and 4) expected heterozygosity (H_E).

RESULTS AND DISCUSSION

Description of microsatellite loci

Seventeen loci did not amplify while 38 displayed multiple bands (Table 2). We did not test these loci further. Loci Bgel_006, Bgel_011, Bgel_031, Bgel_041, Bgel_047, and Bgel_058 had multiple peaks on the capillary sequencer.

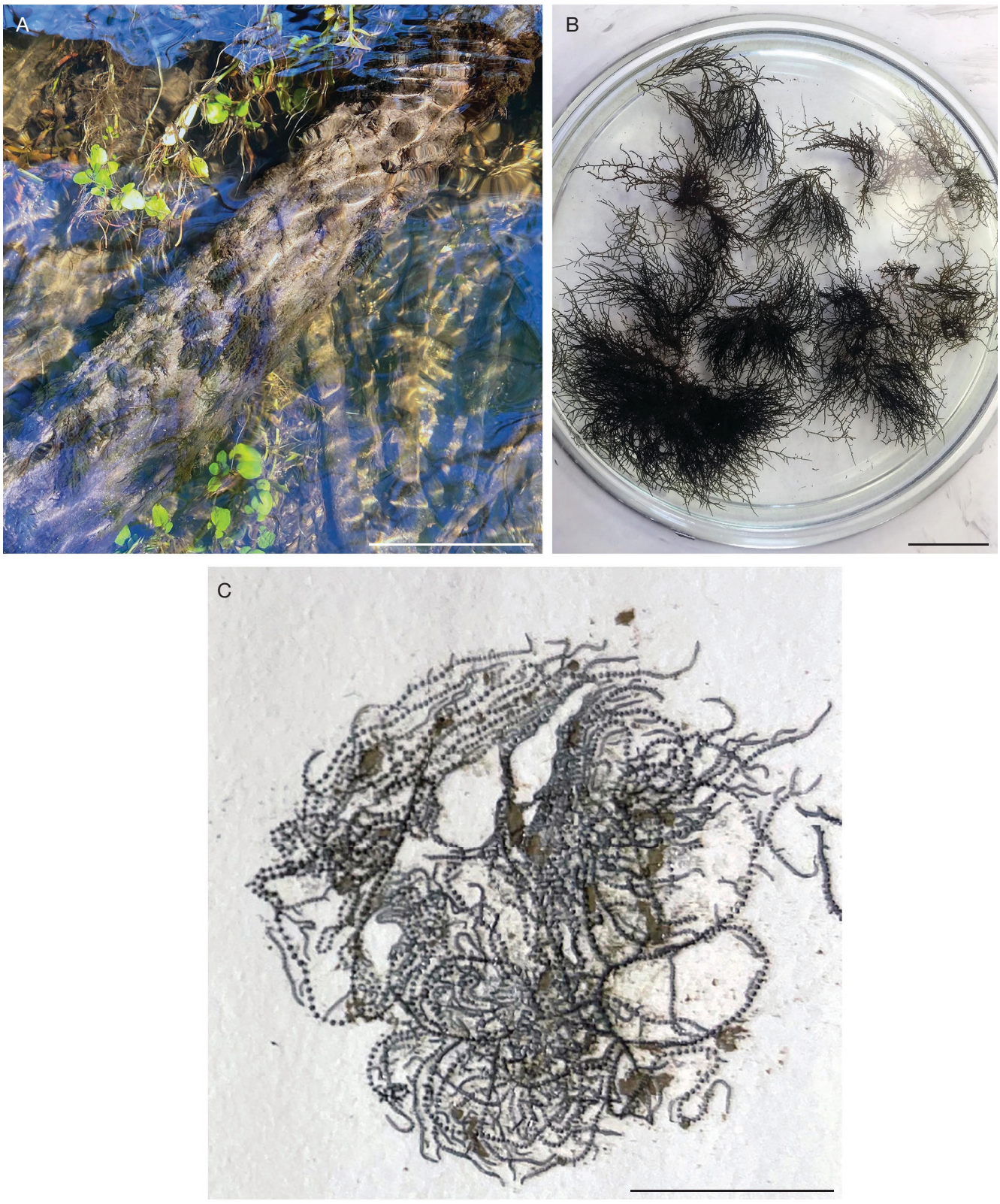

Fig. 1. — Images of *Batrachospermum gelatinosum* (L.) De Candolle: **A**, gametophytes fixed to a log at Cedar Bog in Ohio. Often this species has a brown to olive-green color; **B**, multiple gametophytes in a 55 cm diameter bowl; **C**, a single gametophyte mounted on herbarium paper. Photo credits: A, Stacy A. Krueger-Hadfield; B, C, Morgan L. Vis. Scale bars: A, 10 cm; B, 1.5 cm; C, 1 cm.

TABLE 2. — Microsatellite locus information for *Batrachospermum gelatinosum* (L.) De Candolle. Locus name, repeat motif, expected size, oligo sequences, agarose gel amplification profile, fluorochrome used on the forward oligo, and fragment analysis (**FA**) amplification profile. Note: fluorochrome and FA profile columns are only for loci tested on the capillary sequencer. **Diallelic[1]**, we consistently observed two alleles when only one should be present in a haploid gametophyte.

Locus	Motif	Exp. size	Oligo sequences	Gel profile	Fluorochrome	FA profile	Allele size range	Total alleles
Bgel_021	AGG	144 bp	F: GTTTCGAAGCTCAGTGTCCG R: GGAATTCTCGACGCACTTGG	Single	6-FAM	Single	138-141	2
Bgel_052	AT	312 bp	F: GGGTCAATGCAAGTGGATGG R: AGACCTTGGAAGCTACGACG	Single	VIC	Single	310-312	2
Bgel_053	AG	327 bp	F: GCTGTCATGTCGCCAGAATG R: GGAAGATGCACCTTTGGACG	Single	VIC	Single	298-328	6
Bgel_056	AG	273 bp	F: CTTGCTCACGACTTTGGACC R: CGGAGTGAAACGAAACGAGG	Single	VIC	Single	272-284	5
Bgel_057	AGC	396 bp	F: GCTGAATGAGGTGATGTGGC R: TGCACGTGGTTCTTGACAAG	Single	PET	Single	382-412	7
Bgel_059	AGG	272 bp	F: TTTGAGTACCACCACCCGTC R: GGGAAGTAGGCGTAGAAGGG	Single	VIC	Single	252-258	2
Bgel_067	AG	191 bp	F: AGGCCAACATGCAGCAATAG R: CAAGTTGCTTTGTTCGCTGC	Single	6-FAM	Single	178-182	3
Bgel_070	AG	130 bp	F: TGGAGGCTAACGACATGGAC R: CCGCACAAAGTAGTCGATCG	Single	6-FAM	Single	120-140	7
Bgel_071	AG	226 bp	F: TTATCCACTCCCGGTCTTGC R: GTTTGAAGCGTGGGAAGAGG	Single	NED	Single	215-299	14
Bgel_073	ACG	294 bp	F: TCGACTTTGCAAACTCCAGC R: GGTACGTGTGGACAAACGAC	Single	VIC	Single	272-308	6
Bgel_035	ACG	424 bp	F: GTTGGCGGAAATGGAGTGAG R: CTTCGACATCATGCTGAGCG	Single	PET	Diallelic[1]	–	–
Bgel_048	AG	348 bp	F: AACTTGGCCACGCATTCATC R: CAATGGTCATCTGCCGTGTC	Single	PET	Diallelic[1]; poor amp.	–	–
Bgel_006	ATC	200 bp	F: CTCGTTCAAAGCTAGGCGTG R: TAAACAGGCCCTATGTCCGG	Single	NED	Multiple	–	–
Bgel_011	AGG	261 bp	F: CTGCTTCGACACCAACGTAC R: TCTCTGCCTCTCCATTCACG	Single	NED	Multiple	–	–
Bgel_031	AGCC	372 bp	F: CTCTGGTGCGCTGTTTATCG R: ACCAAACGGAAACAGCTGAC	Single	PET	Multiple	–	–
Bgel_041	AAC	211 bp	F: TGAAGGCGTTGTTGGGAAAC R: GGTGGATTCAAGCGCCTATC	Single	NED	Multiple; poor amp.	–	–
Bgel_047	AAT	183 bp	F: TAAGGTCGCTTCTCCACCAC R: ATTCAAGCCTTCGAAGCTGC	Single	6-FAM	Multiple	–	–
Bgel_058	AG	367 bp	F: CCGTTTCTGCAGTCGTCATC R: CCTGAAGCTGCTGGAAATCG	Single	PET	Multiple	–	–
Bgel_001	AAC	292 bp	F: GTTGACCGGTGTTCAAGTCG R: GATTCGTCGCTTCGGAATCC	Multiple	–	–	–	–
Bgel_002	AAC	166 bp	F: GCGGACACAAAGCAGTAGAC R: ACAACAACGACAATGGACCG	Multiple	–	–	–	–
Bgel_003	AGC	241 bp	F: ACAGGAGTATGCAGAACCGG R: GAAAGCTGCACTCCACCATC	Multiple	–	–	–	–
Bgel_004	AGG	255 bp	F: AGTACACGAGCCACCATCTC R: TGAGAGGAAGCAGCAGTCAC	Multiple	–	–	–	–
Bgel_005	AAC	231 bp	F: GTGGAGCCAACACGTTACG R: TCCTGGAGTGTTACTGGCTG	Multiple	–	–	–	–
Bgel_007	AAG	141 bp	F: GGTGCTGGTTGATTGATGGG R: TGAGAACGAGGAGGCCAATC	Multiple	–	–	–	–
Bgel_008	AGC	308 bp	F: TTCGGTTCCGGTTCTACTCC R: GTCTTCCGTCTTTGCCATCG	Multiple	–	–	–	–
Bgel_009	ATC	273 bp	F: GCGTAATGGTGGTGGTCAAG R: ACCACTGGACGAGATGACTG	Multiple	–	–	–	–
Bgel_010	AG	277 bp	F: AGGCAGTTATCTTCCCGACC R: CACCGGATACTGACGTTTGC	Multiple	–	–	–	–
Bgel_012	AGC	346 bp	F: ACCACCTAGTTCTGCACCTC R: GACGATGCATGCGAGAGATG	Multiple	–	–	–	–
Bgel_013	AGC	302 bp	F: TGAAGGAGGCAGAGATGAGC R: CGTTCATCACTCGCGAAGAC	Multiple	–	–	–	–
Bgel_014	AGC	130 bp	F: GCAATATGAGGCAGCAAGGG R: TCTCAGCACGACACACTACC	Multiple	–	–	–	–
Bgel_015	ATC	167 bp	F: AGTGGATTGATTGTGCTGCG R: CCATCTTCGGTGCGTCTTTC	Multiple	–	–	–	–
Bgel_016	AG	213 bp	F: ACGGCGATGATTTGTTTCCC R: AACAGTACTCCGCTCTCGTC	Multiple	–	–	–	–

Table 2. — Continuation.

Locus	Motif	Exp. size	Oligo sequences	Gel profile	Fluorochrome	FA profile	Allele size range	Total alleles
Bgel_017	AAG	176 bp	F: TCCTTTCTCCTCCTCGCAAC R: GACCTGGACGTTTGAATCGC	No amp.	–	–	–	–
Bgel_018	AAG	290 bp	F: GCACAGATACATTCGGCGTC R: TCCCATCGAACATCCACCTC	Multiple	–	–	–	–
Bgel_019	AT	241 bp	F: GTACTTATGTGCGGCCTTGG R: CAGTCCCGGTCTATGTAGGC	Multiple	–	–	–	–
Bgel_020	AG	315 bp	F: TAGAATGAGACGGGCGATCG R: CCGCTTTGAGTCCGTAAACC	Multiple	–	–	–	–
Bgel_022	AC	298 bp	F: GCCATCCTCTTGCCACATTC R: GTTTGGGTTCCGTCTGTCAG	No amp.	–	–	–	–
Bgel_023	ACT	161 bp	F: TGTCGACCATAAGCTCCGAG R: GTCACCTGGGCAAGCATTAC	No amp.	–	–	–	–
Bgel_024	AAC	236 bp	F: TTGCGCAGATTCACGAACTG R: AGGTGATAAGAGGCGGTGAC	Multiple	–	–	–	–
Bgel_025	ACT	244 bp	F: TGAGTGATTGCGGCCATTTC R: AGTGGCACCTCGATATACCG	No amp.	–	–	–	–
Bgel_026	AGC	282 bp	F: TCTGATGGTAGGGTTGCTGG R: AGAGGGCTGTAGTGAATCGG	Multiple	–	–	–	–
Bgel_027	AGG	190 bp	F: ATCGGTCAAGAGTTGCATGC R: ACGTCTCTATTCCATCGCCC	Multiple	–	–	–	–
Bgel_028	AC	389 bp	F: ATTGCTCCGTCATTGGCATG R: ACTCACACCACACTCCGTAG	No amp.	–	–	–	–
Bgel_029	ACTC	330 bp	F: TCGCGCTCATTTCCAAACTC R: TCAGTCGATCAAGGAGCTGG	Multiple	–	–	–	–
Bgel_030	AAT	280 bp	F: GCTGCGTCACTCTTCTCATG R: TTTCCTCTTGTTGTGCTCGC	No amp.	–	–	–	–
Bgel_032	ATC	226 bp	F: GATTCCAAATACCACCGGCG R: ATCGCCTGGGATGATCGATC	Multiple	–	–	–	–
Bgel_033	ACGG	159 bp	F: CCTGCACTTGTGACGATTCC R: GGACGCTTCGAAGGAACATC	No amp.	–	–	–	–
Bgel_034	ATC	281 bp	F: CGTCGTCGTCAATGTTCCTG R: CCTTGCTGTGGAACTTGGTG	Multiple	–	–	–	–
Bgel_036	AC	179 bp	F: TCGTCCTGGTCCATGCTAAG R: CCTGCCCGTTTGCTTATGAG	No amp.	–	–	–	–
Bgel_037	AAC	179 bp	F: CCTCCCAACGAAACATCAGC R: ATTACGAGTGTCACCGGGAG	No amp.	–	–	–	–
Bgel_038	AGAT	159 bp	F: CTATTTCGATTCGTCGCGGG R: AAGACAGAACCTCCGCTCAG	Multiple	–	–	–	–
Bgel_039	AAT	148 bp	F: CCACTTCGGTTTCAGGAAGC R: CGGTCAGAATCATCACGTGC	No amp.	–	–	–	–
Bgel_040	ATC	140 bp	F: TCCTCTCCATTCAGCAGTCG R: CAAGAGAAGCTGAAGACGCG	Multiple	–	–	–	–
Bgel_042	AC	418 bp	F: TGGACATACTCGCTCCACAG R: GCAGCGCTTAGAGTGTGAAG	No amp.	–	–	–	–
Bgel_043	AAT	275 bp	F: TCCAACTCCTCAACGACCTG R: TTGGAGCAGAATTCGTTCGC	Multiple	–	–	–	–
Bgel_044	AAC	333 bp	F: AGCCAATACCAACCCTCGAG R: TGCATCATTCAACTCGCCAC	Multiple	–	–	–	–
Bgel_045	AAC	239 bp	F: ACCGAACAAGCACTTCAACC R: ATGACGTCGTTGGCAAGAAC	Multiple	–	–	–	–
Bgel_046	AAC	173 bp	F: CAGCAGCGAGTGGAAAGTAC R: GCTACACGAAGATGGGCAAC	Multiple	–	–	–	–
Bgel_049	AATT	395 bp	F: GTTCGACGGTCATCAGCATG R: TCTTCCAACCCTGCTCTGAC	No amp.	–	–	–	–
Bgel_050	AGG	251 bp	F: AAGTGGAGGAGGAATGGGTG R: ACCTCTGCCTCACTCATTCC	No amp.	–	–	–	–
Bgel_051	AAT	147 bp	F: CATCATATCCCTGCCCTCCC R: AATGCAGCCATGACTCGTTG	Multiple	–	–	–	–
Bgel_054	AC	199 bp	F: TGCGTTGTCTTCTTTGCTCC R: CATCCGCCACAGAAACCATG	No amp.	–	–	–	–
Bgel_055	AC	282 bp	F: TCAGAGGAATGGATGGACGC R: GATGTTCCGTGCAGATCAGC	Multiple	–	–	–	–
Bgel_060	AAG	226 bp	F: CTTTCTTCACCGACGCTGAC R: CCGGGCTCAGAAATTTCGTC	No amp.	–	–	–	–
Bgel_061	AAAT	137 bp	F: CAGATGATTCGAGCGATGCC R: CACGGGCATGACAAATCTCC	No amp.	–	–	–	–

Table 2. — Continuation.

Locus	Motif	Exp. size	Oligo sequences	Gel profile	Fluorochrome	FA profile	Allele size range	Total alleles
Bgel_062	AG	228 bp	F: CGAACAACAGACATAGCGGG R: TCTTCCTGCGGCTGTAAGAG	No amp.	–	–	–	–
Bgel_063	AT	148 bp	F: GTTCACCTGTGGAAGCGAAG R: AGTAATCGTCTCGCCTGGTG	Multiple	–	–	–	–
Bgel_064	AAG	315 bp	F: GCTTTGACCGAGTTGTACCG R: CGATTAGGCGGTTGTGTTGG	Multiple	–	–	–	–
Bgel_065	AAG	226 bp	F: GTTGACGACGCTTCATCGAG R: TGGGACTTCGAGTGGTTGAG	Multiple	–	–	–	–
Bgel_066	AAC	157 bp	F: TACACGTGTGAGGAGGCTTC R: ATTCACATCATCACCAGCGC	Multiple	–	–	–	–
Bgel_068	AGC	139 bp	F: TGCTGGACTAGTGACAGTGG R: TGCCTCACTACTGTCACCAC	Multiple	–	–	–	–
Bgel_069	AGC	217 bp	F: TGGGAGAAGACGAGCTGATG R: GGCTCAGAATCATGCTGACG	Multiple	–	–	–	–
Bgel_072	AAG	390 bp	F: GCCATCTTCATACGTCGCTG R: ACTTTGATCAGGCTCTCGGG	Multiple	–	–	–	–

Multiple peaks are likely due to non-specificity in the primer binding sites that were not easily distinguished on agarose gel electrophoresis rather than contamination of multiple gametophytes. As we had other promising loci, we did not try to optimize any of these loci further. Loci Bgel_035 and Bgel_048 had two alleles for many gametophytes, suggesting the amplification of more than one locus (see also Krueger-Hadfield *et al.* 2011). The remaining ten loci (Table 2) had a single allele per gametophyte, as expected, and were used to genotype the gametophytes collected from Cripple Creek.

Preliminary population genetic analyses at Cripple Creek

We observed null allele(s) at low frequency at a single locus Bgel_067 (3.6%, only one out of 28 gametophytes; Table 3). The remaining loci amplified at all gametophytes from Cripple Creek. The *pid* value was 0.004, suggesting some resolution to distinguish among gametophytes with these ten microsatellite loci. These loci will be useful for future population genetic analyses in *B. gelatinosum* due to the low frequency of null alleles and levels of polymorphism with which to distinguish among genotypes in a population.

Of the 28 gametophytes genotyped, we observed 16 distinct genotypes at Cripple Creek. Four of these genotypes were encountered more than once: one genotype was re-encountered nine times, one three times, and two twice. Thus, genotypic richness was moderate ($R = 0.556$). Multilocus genotypes were dispersed throughout the reach we sampled at Cripple Creek. Correspondingly, genotypic evenness was high ($D^* = 0.892$). There was little evidence of linkage disequilibrium (= 0.032). However, five of the ten loci at this site were fixed, in which all sampled gametophytes had the same allele, thereby potentially decreasing . While we only found one allele at Cripple Creek, we note we found other alleles in our initial screening of gametophytes including other sites throughout the *B. gelatinosum* range. Genetic diversity was low ($H_E = 0.161$).

Together, these data hint at the prevailing reproductive mode of *B. gelatinosum* at Cripple Creek. Repeated genotypes typically are interpreted as a signal of asexual reproduction. It is highly unlikely that repeated gametophytes are due to fragmentation and reattachment of gametophytes along a reach as gametophytes are physically connected to the chantransia. Other studies have observed repeated gametophytic genotypes in marine red macroalgae and suggested that this finding was due to limited resolution of the markers, compounded by the presence of only one allele per locus in the haploid phase (e.g. Guillemin *et al.* 2008; Lees *et al.* 2018). It is possible that these loci are not sufficiently polymorphic at this site, though the *pid* value was moderate suggesting resolution among individual genotypes.

Repeated genotypes could be the result of monospore production by the chantransia. Monospore production could generate a pattern of repeated chantransia genotypes throughout a stream reach. We have yet to genotype chantransia and the level of heterozygosity is thus unknown. However, we would not expect monospore production to lead to repeated gametophytic genotypes spread over *c.* 45 m of stream sampled at Cripple Creek. Asexuality tends to lead to an excess of heterozygosity (Balloux *et al.* 2003), and this has been shown in other red macroalgae (e.g. Guillemin *et al.* 2008; Krueger-Hadfield *et al.* 2016). Even if a single chantransia network of cells covered an entire rock, we would expect unique gametophytic genotypes because of meiosis and recombination and the novel combination of alleles at different loci from heterozygous, diploid chantransia. Instead, repeated genotypes could be due to intragametophytic selfing in which the union of a spermatium and a carpogonium from the same gametophyte would result in instantaneous, genome-wide homozygosity for the resultant carpospores. Carpospores would likely settle near one another, but even if carpospores dispersed throughout a reach, they would produce identical gametophytic genotypes as all loci will have two copies of the same allele, barring mutation.

Future perspectives

We now need to use these ten loci to genotype *B. gelatinosum* gametophytes from across the North American range. It is

TABLE 3. — Null allele frequencies for each locus were determined by non-amplification after two or three PCR attempts for gametophytes from Cripple Creek, Alabama. As gametophytes are haploid, non-amplification of an allele at a given locus was considered a null allele (see also Krueger-Hadfield *et al.* 2013).

Locus	Null Allele Frequency (%)
Bgel_021	0.0
Bgel_071	0.0
Bgel_052	0.0
Bgel_067	3.6
Bgel_053	0.0
Bgel_070	0.0
Bgel_059	0.0
Bgel_073	0.0
Bgel_057	0.0
Bgel_056	0.0

unclear if patterns at Cripple Creek, near the lower latitudinal range limit for the species are representative of the general pattern. Moreover, as we only genotyped the gametophytic phase of the life cycle, we are correspondingly limited in the types of summary statistics that are possible to calculate in order to describe the reproductive system (e.g. F_{IS}) at present. Thus, future studies should also include temporal sampling to compare genotypic frequencies across generations (e.g. Becheler *et al.* 2017), especially as the gametophytes are ephemeral in freshwater reds. Moreover, future sampling and genotyping efforts should include the chantransia, but developing methods to genotyping microscopic phases in life cycles are challenging (see discussion in Schoenrock *et al.* 2021). Nevertheless, these loci constitute an important addition to the available genetic resources for freshwater algae and will enhance our understanding of macroalgal population dynamics.

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Authors contribution

R.M. Crowell and S.J. Shainker-Connelly are joint first authors; authorship order determined by a coin toss.

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