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Sarah Shainker-Connelly, Solenn Stoeckel, Morgan Vis, Stacy Krueger-Hadfield

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RESEARCH ARTICLE

Monoicy, dioicy, and genetic structure in three species of *Sheathia* (Batrachospermales, Rhodophyta)

Sarah J. Shainker-Connelly[1] | **Solenn Stoeckel**[2] | **Morgan L. Vis**[3] | **Stacy A. Krueger-Hadfield**[1,4,5]

[1]Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA

[2]DECOD (Ecosystem Dynamics and Sustainability), INRAE, Institut Agro, IFREMER, Rennes, France

[3]Department of Environmental and Plant Biology, Ohio University, Athens, Ohio, USA

[4]Virginia Institute of Marine Science Eastern Shore Laboratory, Wachapreague, Virginia, USA

[5]William and Mary's Batten School of Coastal and Marine Science, Gloucester Point, Virginia, USA

Correspondence
Stacy A. Krueger-Hadfield, Virginia Institute of Marine Science Eastern Shore Laboratory, Wachapreague, VA 23480, USA.
Email: sakh@vims.edu

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Abstract

Sexual systems (i.e., separate vs. combined sexes) vary widely among eukaryotes and influence the evolution of reproductive systems, which shape genetic structure and evolutionary trajectories. In diploid-dominant angiosperms, combined (i.e., hermaphroditism) and separate sexes are expected to correlate with selfing and outcrossing, respectively. When sex is determined in the haploid phase, selfing is possible even when there are separate sexes. The freshwater red macroalgal genus *Sheathia* (Batrachospermales) displays sexual system variation within and among populations, but no prior data exist on the reproductive systems of these populations. We developed 16 polymorphic microsatellite loci to characterize the reproductive system and genetic structure of three *Sheathia* species. We observed cross-amplification of loci across the three targeted species, suggesting these markers may be useful in other *Sheathia* spp. We observed variation in monoicy (i.e., hermaphroditism) versus dioicy (i.e., separate sexes) in each species, including *S. americana*, which was previously believed to be obligately dioicous. Our data suggest that *S. americana* and *S. involuta* display more variation in their prevailing reproductive modes as compared to *S. grandis*. Generally, dioicy resulted in greater diversity in contrast to monoicy. We observed strong population structure that is likely driven by uniparental reproduction and limited dispersal; however, there is limited population connectivity that may be facilitated by long-distance dispersal events. Overall, these data contribute to our knowledge of the relationship between the sexual system, reproductive system, and population genetic structure in haploid-diploid taxa, thereby informing a broader understanding of the evolution of sex.

KEYWORDS
evolution, gene flow, population genetics, sex, sexual system, streams

Abbreviations: BHO, Bartley Herbarium at Ohio University; BIC, Bayesian information criterion; BSA, bovine serum albumen; DAPC, discriminant analysis of principal components; dNTP, deoxynucleoside triphosphate; GPS, global positioning system; LD, linkage disequilibrium; MLG, multilocus genotype; PC, principal components; PCR, polymerase chain reaction; *pid*, probability of identity between pairs of individuals; SSR, single sequence repeat.

INTRODUCTION

In many eukaryotes, sexual systems (i.e., separate vs. combined sexes) can vary widely within and among species (Ashman et al., 2014; Barrett, 2002). Separate versus combined sexes influence the reproductive system (i.e., the relative rates of sexual vs. asexual reproduction and selfing versus outcrossing; Barrett, 2011). Most studies of sexual- and reproductive system variation have focused on organisms with long-lived diploid phases, such as animals and angiosperms (note angiosperms can be considered ecologically diploid). In angiosperms, sex is determined in the diploid stage, and many lineages exhibit evolutionary transitions between monoecy (i.e., hermaphrodites, combined sexes) and dioecy (i.e., separate sexes) that correlate with switches between self-fertilization (i.e., selfing) and outcrossing, respectively (Barrett, 2002). Selfing leads to the accumulation of deleterious mutations, but once these mutations have been purged from a population, monoecy is advantageous when the probability of successful outcrossing is limited, such as during the colonization of new habitats and when adapting to marginal or stressful environments (Barrett, 2011). Dioecy is associated with outcrossing, which can generate genetic diversity (Barrett, 2002). It was previously thought that trioecy (i.e., populations with monoecious, male-only, and female-only individuals) was a transitional state and that all lineages would evolve toward either obligatory hermaphroditism or separate sexes (Lande & Schemske, 1985). However, more recent models have shown that trioecy can be an evolutionarily stable state (Anderson et al., 2020).

Although most of our knowledge of sexual-system variation comes from research focused on the diploid-dominant angiosperms, many eukaryotes—including some macroalgae—have life cycles in which sex is determined in the haploid phase (Bell, 1994). Selfing is thought to be more advantageous in these haploid or haploid-dominant taxa compared to diploid and diploid-dominant taxa because mutations in the haploid phase are directly exposed to selection and can be purged from a population (Otto & Marks, 1996). In cases where sex is determined in the haploid phase, gametophytes can display the same degree of sexual-system variation as angiosperms. Gametophytes can have combined sexes (i.e., monoicy), separate sexes (i.e., dioicy), or both hermaphroditic and separate sex gametophytes (i.e., trioicy; see Krueger-Hadfield et al., 2024). Similarly to monoecy in diploid-dominant organisms, monoicy in the haploid phase may be beneficial during colonization as only one individual is needed to establish a population (i.e., Baker's Law; Baker, 1955). We note the importance of the differentiation in these terms and spelling as monoicy, dioicy, and trioicy denote sex determination in the haploid phase, while monoecy, dioecy, and trioecy denote sex determination in the diploid phase.

Although macroalgae have been proposed as models to study the evolution of sex due to variation in their sexual systems (Krueger-Hadfield et al., 2024; Otto & Marks, 1996), few studies have focused on these groups (see perspectives by Krueger-Hadfield, 2024; Krueger-Hadfield et al., 2024). Here, we have focused on freshwater red macroalgae in the order Batrachospermales, which exhibit notable variation in their sexual system within and among species (Krueger-Hadfield et al., 2024). Batrachospermalean algae alternate between a microscopic, perennial diploid phase—called the "chantransia"—and a macroscopic, ephemeral gametophyte. Gametophytes can be monoicous or dioicous, and each gametophyte remains physically attached to its parental chantransia (Sheath, 1984). This life cycle produces a suite of possible outcomes. Because the two phases remain physically attached and red algal propagules are non-motile (Searles, 1980), dispersal is likely limited. Chantransia undergo vegetative meiosis in which they produce physically attached gametophytes, unlike other red algal groups in which meiosis produces haploid spores that can disperse before producing a morphologically independent gametophyte (Sheath, 1984). Chantransia can reproduce asexually by producing monospores, which develop into new chantransia (Sheath, 1984). In monoicous species, intragametophytic selfing describes fertilization between a spermatium and a carpogonium produced by the same monoicous gametophyte, resulting in instantaneous, genome-wide homozygosity (Klekowski, 1969; see figure 1a in Shainker-Connelly et al., 2024). Intergametophytic selfing occurs between two gametophytes that share the same parental chantransia. It is possible that multiple gametophytes may be produced from different filaments of the same chantransia. These gametophytes would be located near one another, potentially facilitating intergametophytic selfing, which is expected to gradually erode genetic diversity like selfing in diploid-dominant organisms (Klekowski, 1969; Shainker-Connelly et al., 2024). This form of selfing involves cross-fertilization and is not possible in diploid, dioecious taxa. For Batrachospermalean algae, we considered uniparental reproduction to encompass both forms of selfing and monospore production by the chantransia. Conversely, outcrossing, or intergametophytic crossing (Klekowski, 1969), describes fertilization between gametophytes that have genetically distinct chantransia parents (see figure 1b in Shainker-Connelly et al., 2024). Outcrossing is expected to generate and maintain heterozygosity (see Goodwillie et al., 2010; see figure 1c in Shainker-Connelly et al., 2024). Previous work characterizing the reproductive system of

the widespread, monoicous freshwater red alga *Batrachospermum gelatinosum* determined intragametophytic selfing as the prevailing reproductive mode (Shainker-Connelly et al., 2024), which along with limited dispersal, generated strong genetic structure in which regions and sites were genetically isolated from one another (Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024).

The genus *Sheathia* is widely distributed in temperate and tropical regions (Salomaki et al., 2014; Vis & Necchi, 2021). Most species have both monoicous and dioicous populations, but some are strictly monoicous or dioicous; others are trioicous (Krueger-Hadfield et al., 2024; Salomaki et al., 2014; Vis & Necchi, 2021). Gametophytes and chantransia can be seen in freshwater streams on hard substratum such as rocks, logs, or tree roots (Sheath & Vis, 2015; Vis & Necchi, 2021). Many species can only be distinguished through molecular markers, such as the *rbc*L gene (Salomaki et al., 2014), rather than morphology, likely inhibiting earlier studies of reproductive system variation as some taxonomic designations are still in progress. We chose three focal species—*S. americana, S. grandis*, and *S. involuta*—for which we sampled enough gametophytes at the site level (i.e., putative population) to characterize the reproductive system. We chose *S. americana* as a focal species because prior studies have indicated it is obligately dioicous, providing a foil to the previously characterized monoicous species *Batrachospermum gelatinosum* (see Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024; Shainker-Connelly et al., 2024). *Sheathia americana* is mostly distributed in New England (Salomaki et al., 2014; Vis & Necchi, 2021). We opportunistically encountered *S. grandis* and *S. involuta*. These species have both monoicous and dioicous populations, and we chose to include them as congeneric comparisons to *S. americana*. *Sheathia grandis* is mostly distributed in the midwest United States (Salomaki et al., 2014; Vis & Necchi, 2021). *Sheathia involuta* is the most widespread of the three throughout North America, and there are even a few isolated populations in Europe (Salomaki et al., 2014; Vis et al., 1996; Vis & Necchi, 2021).

We sampled gametophytes from *Sheathia americana*, *S. grandis*, and *S. involuta* from 22 stream sites to characterize the species' sexual systems, reproductive mode, and genetic structure across the eastern United States. We measured environmental stream conditions and characterized the sexual systems in each site by identifying reproductive structures on sampled gametophytes. To assess their reproductive mode and genetic differentiation, we developed microsatellite loci for *S. americana* and *S. grandis* and cross-amplified them in *S. americana*, *S. grandis*, and *S. involuta* gametophytes. (See Methods as to why we have genotyped the gametophyte phase only.) We used 16 of these microsatellite loci to genotype the sampled gametophytes and compute genetic analyses that allowed estimates of rates of clonality and selfing within sites and genetic structure between sites. We used these environmental, sexual-system, and genetic measures to understand the biology of these three *Sheathia* species in the eastern United States and to improve our understanding of the relationship between sexual-system and reproductive-system variation in haploid-diploid freshwater Rhodophyta. We have grouped our hypotheses into those that characterize reproductive-system variation and those that describe genetic structure.

Regarding the reproductive system, we hypothesized that the dioicous species *Sheathia americana* would exhibit greater genetic and genotypic diversity compared with *S. grandis* and *S. involuta* because the latter two species include monoicous populations. We expected that *S. involuta* and *S. grandis* would exhibit greater reproductive system variation, driven by variation in monoicy and dioicy across sites as compared to dioicous populations of *S. americana*. Specifically, we expected that monoicous populations would be characterized by more selfing or asexual reproduction (see discussion in Shainker-Connelly et al., 2024 about the difficulty in easily distinguishing between these two reproductive modes) than dioicous populations would be, regardless of species.

Regarding genetic structure, we hypothesized patterns of strong differentiation among sites and predicted that this differentiation would rapidly increase with increasing geographic distance, due to the limited dispersal capabilities of freshwater red algae; however, there may be possible, occasional instances of passive dispersal. For example, Hall and Vis (2002) observed that populations of the freshwater red alga *Virescentia viride-americana* (as *Batrachospermum helminthosum*) were genetically distinct and that genetic variation was not partitioned by geographic distance, suggesting that there may be some occurrences of long-distance dispersal. If dispersal is driven by water flow, we would expect to see genetic clustering by watershed. If dispersal is zoochorous (i.e., animal-mediated)—for example, via adhesion to waterfowl (Kristiansen, 1996)—we would expect to see more stochastic genetic clustering that would not necessarily be correlated with drainage basin nor geographic distance. We note that we were exploring contemporary patterns of gene flow that have also been influenced and structured by earlier vicariant events during the last glacial maximum (see also Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024). Together, these two classes of hypotheses allowed us to explore how the sexual system and the reproductive system structure *Sheathia* spp. populations in eastern North America, setting the stage for future work to explore these mechanisms in freshwater red algae. These data are integral to expanding our

understanding of the evolution of sex across organisms with diverse life-cycle types.

MATERIALS AND METHODS

Sample collection

Due to the diminutive size of the chantransia, population genetic work in the Batrachospermales has been limited to the gametophytic phase (see Chiasson et al., 2003; Crowell, Shainker-Connelly, Vis, & Krueger-Hadfield, 2024; Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024; Hall & Vis, 2002; Shainker-Connelly et al., 2024, 2025; Vis et al., 2008). There are limitations on the analyses we can perform with haploid data: measures of heterozygosity are meaningless (e.g., observed heterozygosity, H_O; inbreeding coefficient, F_{IS}), and recent developments in population genetic theory, such as temporal analyses of genotype frequency (e.g., Becheler et al., 2017), are not yet possible. There are promising techniques that can allow us to sample microscopic phases, such as the microsatellite pooled sequencing methods implemented in Wolf et al. (2021), but this technique for chantransia would only have allowed a description of allele frequencies, precluding other analyses typical for studying the reproductive system, such as estimating heterozygosity. These difficulties arise because all downstream analyses assume a single genotype in a sample and cannot be easily completed on complex environmental samples that contain many genotypes. Chantransia are too small to easily separate into "individuals" useful for population genetic analyses as undertaken in other red algae with macroscopic sporophytes and gametophytes (e.g., *Gracilaria*, Engel et al., 2004; *Chondrus*, Krueger-Hadfield et al., 2013). Analogous problems exist in kelps, in which only the macroscopic sporophytic phase has been included in all population genetic surveys to date (e.g., Alberto et al., 2011; Billot et al., 2003; Robuchon et al., 2014; Vranken et al., 2022), but this does not negate the overall importance of the bank of microscopic forms in algae (see Hoffmann & Santelices, 1991; Schoenrock et al., 2021). For this study, we only collected and genotyped macroscopic gametophytic thalli (henceforth referred to as "gametophytes").

We collected *Sheathia* spp. gametophytes from 20 sites throughout the eastern United States in 2021 and 2022: *S. americana* from five sites, *S. grandis* from nine sites, and *S. involuta* from six sites (Figure 1, Table 1). For each site, we noted the GPS coordinates with Google Maps or the iPhone app GPSCoordinates ver. 5.18 (Neal, 2018). On the collection date, we measured stream width and length of the sampled stream segment with a transect tape and measured physiochemical parameters close to the middle of the

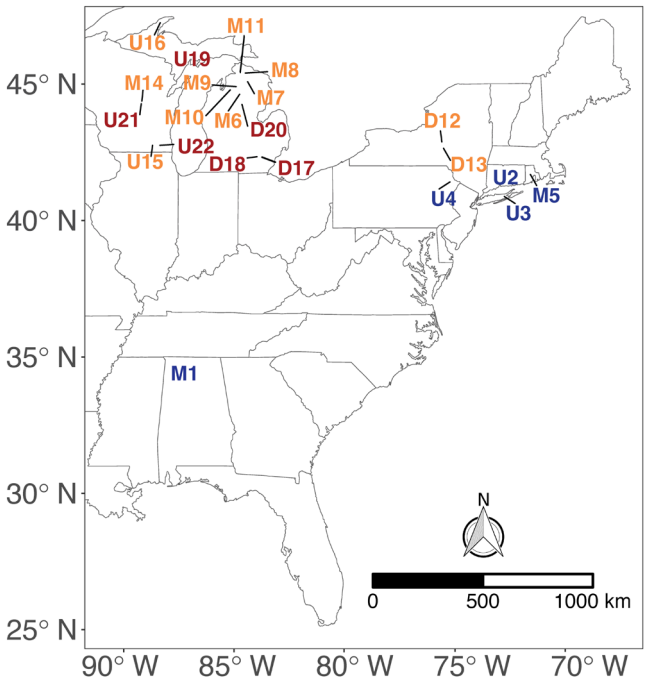

FIGURE 1 Map of collection sites for *Sheathia americana* (blue), *S. grandis* (yellow), and *S. involuta* (red) gametophytes for eastern North America. We sampled a total of 22 sites throughout eastern North America: Five *S. americana* sites (site numbers 1–5; blue), 11 *S. grandis* sites (site numbers 6–16; orange), and six *S. involuta* sites (site numbers 17–22; red). Names, abbreviations, and numbers associated with each site are shown in Table 1. The letter preceding the site name indicates the sexual system: D, "dioicous"; M, "monoicous"; U, "unknown".

sampling area. We estimated stream bed composition, water color, and water clarity (Table S1). At sites Sa-M1, Sa-U2, Sa-U3, Sa-U4, Sa-M5, Sg-M14, Si-D17, Si-U19, and Si-D20 (see Table 1), we used an Oakton PCTSTestr 50 Pocket Tester to measure pH, water temperature, and specific conductivity. At sites Sg-M6, Sg-M7, Sg-M8, Sg-M9, Sg-M10, Sg-M11, Sg-U15, Sg-U16, and Si-D18, we used an Oakton pHTestr 5 to measure pH and water temperature and an Oakton ECTestr, Low to measure specific conductivity. At sites Sa-M1, Sa-U2, Sa-U3, Sa-U4, Sa-M5, Sg-M14, Si-D17, Si-U19, and Si-D20 (see Table 1), we used a flow probe (Global Water Instruments, Model FP111) to measure current velocity and stream depth. At sites Sg-M6, Sg-M7, Sg-M8, Sg-M9, Sg-M10, Sg-M11, Sg-U15, Sg-U16, and Si-D18, we used another flow probe (General Oceanics, Mechanical Flow Meter) and measured stream depth with a ruler. At sites Sa-M1, Sa-U2, Sa-U3, Sa-U4, Sa-M5, Sg-M14, Si-D17, Si-U19, and Si-D20 (see Table 1), we used a spherical densiometer (Forest Densiometer, Model A) to calculate percent canopy cover following Lemmon (1956, 1957) but with one reading instead of four. At sites Sg-M6, Sg-M7, Sg-M8, Sg-M9, Sg-M10, Sg-M11, Sg-U15, Sg-U16, and Si-D18, canopy cover was estimated by eye. Abiotic parameters were not measured at sites Sg-D12 and Sg-D13.

TABLE 1 Locations and physiochemical data measured at each site in which *Sheathia* sp. gametophytes were sampled.

Site	Site name	Site abbreviation	Latitude	Longitude	Date	Species	Sexual system (F, FM, M)	Herbarium voucher and Genbank accession number	pH	Specific conductivity ($\mu S \cdot cm^{-1}$)	Water temperature (°C)	Current velocity ($m \cdot s^{-1}$)	Stream depth (cm)	Stream width (m)	Sampling length (m)	Canopy cover (%)
Sa-M1	King Spring, Alabama	AL-KIS	34.85700	−87.65400	29-July-2021	*S. americana*	Monoicous (1,2,0)	BHO: A-1909 XXX	6.31	135.3	17	0	16	17.3	38.75	57.2
Sa-U2	Maltby Park, Connecticut	CT-MAL	41.30940	−72.98808	5-Apr-2022	*S. americana*	Unknown (1,0,0)	BHO: A-1907 XXX	7.36	106.1	3.5	–	2	1.3	4.66	34
Sa-U3	Peconic River, New York	NY-PEC	40.90083	−72.77333	4-Apr-2022	*S. americana*	Unknown (4,0,0)	BHO: A-1913 XXX	6.64	65.8	8.8	0.3	38.5	6.1	17.4	0
Sa-U4	Decker Creek, Pennsylvania	PA-DEC	41.43548	−75.15277	1-Apr-2022	*S. americana*	Unknown (2,0,0)	BHO: A-1910 XXX	6.99	109.7	2.4	0.5	13	3.8	5.3	37.2
Sa-M5	Phillips Brook, Rhode Island	RI-PHI	41.63897	−71.74652	6-Apr-2022	*S. americana*	Monoicous (0,1,0)	BHO: A-1911 XXX	5.96	36.1	5.8	0.4	23	2.3	41.5	49.9
Sg-M6	Au Sable River Park, Michigan	MI-ASR	44.65972	−84.71309	12-May-2022	*S. grandis*	Monoicous (4,12,0)	BHO: A-1825 PP235018	8.2	320	20.8	0.3	54	12	6.6	0
Sg-M7	Black River, Michigan	MI-BLA	45.12675	−84.40770	13-May-2022	*S. grandis*	Monoicous (0,10,0)	BHO: A-1830 PP235022	8.37	420	19.7	0.6	17	8.5	15.6	0
Sg-M8	Crumley Creek, Michigan	MI-CLC	45.37473	−84.55086	13-May-2022	*S. grandis*	Monoicous (0,6,0)	BHO: A-1829 PP235021	7.94	370	16.8	0.2	26	0.5	5.4	0
Sg-M9	Manistee River, Michigan	MI-MNR	44.90137	−84.84516	14-May-2022	*S. grandis*	Monoicous (0,4,0)	BHO: A-1836 PP235026	8.16	290	14.3	0.4	90	6	30.2	0
Sg-M10	Rapid River, Michigan	MI-RAP	44.81529	−85.13256	14-May-2022	*S. grandis*	Monoicous (0,4,0)	BHO: A-1837 PP235027	8.56	330	16	0.46	80	6	35.1	0
Sg-M11	West Branch Sturgeon River, Michigan	MI-WSR	45.27163	−84.60154	13-May-2022	*S. grandis*	Monoicous (13,3,0)	BHO: A-1822 PP235015	8.45	420	14.8	1.05	20	8	22	5
Sg-D12	Stream in Hamilton, New York	NY-HAM	42.83398	−75.55934	20-Apr-2023	*S. grandis*	Dioicous (1,0,1)	BHO: A-2070; A-1662 PP139963	–	–	–	–	–	–	–	–
Sg-D13	Rogers Environmental Center in Sherburne, New York	NY-ROG	42.68448	−75.51753	20-Apr-2023	*S. grandis*	Dioicous (0,0,2)	BHO: A-2071; A-1661 PP139962	–	–	–	–	–	–	–	–
Sg-M14	Strait between Lake Orlando and Beasley Lake, near Waupaca, WI	WI-TOM	44.3302203	−89.1825958	9-May-2022	*S. grandis*	Monoicous (0,1,0)	BHO: A-1897 PP235056, PP235057	8.8	405	12	0.05	9	15.15	47.86	36.1
Sg-U15	Bluff Creek, Wisconsin	WI-BLU	42.79898	−88.68417	8-May-2022	*S. grandis*	Unknown (–-)	BHO: A-1888 PP235032-PP235037	8.41	818	10.1	0.1	54	7.7	15.3	0
Sg-U16	Trap Rock River, Michigan	MI-TRR	47.28708	−88.32056	11-May-2022	*S. grandis*	Unknown (1,0,0)	BHO: A-1890 PP235039, PP235040	7.04	32.5	7.5	0.4	81.5	6.6	6.62	0
Si-D17	Matthaei Botanical Gardens (Fleming Creek), Michigan	MI-BOT	42.30200	−83.66010	10-May-2022	*S. involuta*	Dioicous (14,0,2)	BHO: A-1816 PP235011	8.2	740	20	0.75	37	6	37.2	0
Si-D18	Hell Creek, Michigan	MI-HEL	42.43409	−83.98665		*S. involuta*	Dioicous (2,0,2)	BHO: A-1819 PP235014	8.25	500	19.5	0.16	57	6	--	0

TABLE 1 (Continued)

Site	Site name	Site abbreviation	Latitude	Longitude	Date	Species	Sexual system (F, FM, M)	Herbarium voucher and Genbank accession number	pH	Specific conductivity ($\mu S \cdot cm^{-1}$)	Water temperature (°C)	Current velocity ($m \cdot s^{-1}$)	Stream depth (cm)	Stream width (m)	Sampling length (m)	Canopy cover (%)
Si-U19	Rock River, Michigan	MI-HIA	46.38985	−86.91499	14-May-2022	*S. involuta*	Unknown (1,0,0)	BHO: A-1894 PP235050, PP235051	8.3	247	12.3	0.4	43.5	6.77	11.83	5.3
Si-D20	Knappen Creek, Michigan	MI-HLK	44.29844	−84.64934	12-May-2022	*S. involuta*	Dioicous (5,0,3)	BHO: A-1827 PP235020	7.70	190	18.0	–	35	3.4	36.5	0
Si-U21	Hartmann Creek, Wisconsin	WI-HRT	44.32623	−89.20028	8-May-2022	*S. involuta*	Unknown (1,0,0)	BHO: A-1893 PP235048, PP235049	9.09	379	13.8	0.2	14	4.95	26	18.1
Si-U22	Tichigan Creek, Wisconsin	WI-TIC	42.80000	−88.24278	8-May-2022	*S. involuta*	Unknown (1,0,0)	BHO: A-1891 PP235041, PP235046	8.19	870	10.4	0.1	36	3.15	14	0

Note: Dashes indicate that the measurement was not taken. The sexual system column indicates the number of examined thalli that had only female reproductive structures (F), both male and female reproductive structures (FM), and only male reproductive structures (M). BHO = Floyd Bartley Herbarium, Ohio University. GenBank accession number for *rbc*L gene.

We haphazardly sampled 20–30 gametophytes from each site. At some sites, there were few gametophytes with patchy distribution, resulting in smaller sample sizes (Table 2). We observed each gametophyte under a dissecting microscope for the presence of diploid carposporophytes. When possible, we removed these carposporophytes to avoid contamination of haploid samples by diploid tissue. We ensured that we preserved a single gametophyte by physically separating thalli if entangled and removing the lower portion of the thallus to ensure that any remnants of the chantransia and other detritus from the biofilm were removed. We preserved each gametophyte in silica desiccant (Activa Flower Drying Art Silica Gel), and when possible, remaining tissue was mounted on herbarium paper (University of California-type Herbarium Mounting Paper, Herbarium Supply, Bozeman, MT). A representative voucher was deposited in the Floyd Bartley Herbarium at Ohio University (BHO; Table 1).

Determination of the sexual system for each sampled site

Herbarium specimens from each site were rehydrated and examined with a compound microscope for the presence of reproductive structures. In some cases, there was a single voucher gametophyte, and in others, multiple gametophytes could be examined. We distinguished four potential cases:

- If both carpogonia/carposporangia and spermatangia were present on the same thallus, then it was classified as monoicous. If all thalli that could be examined from a site were classified as monoicous, then the site was classified as *monoicous*.
- If some thalli from a site had only spermatangia and other thalli from that same site had only carpogonia/carposporangia or if all thalli from a site had only spermatangia, then the site was classified as *dioicous*.
- If thalli could only be determined as female, then we could not classify the site as monoicous or dioicous with any certainty because of the possibility that the spermatia had already released. We did not designate the sexual system for sites in the latter category, and the site was classified as *undetermined*.
- If both monoicous and dioicous thalli were identified from the same site, then that site would be classified as *trioicous*. We did not observe any trioicous sites in this study.

DNA extraction

We extracted total genomic DNA using the Macherey-Nagel Nucleospin® Plant II kit (Macherey-Nagel)

TABLE 2 Number of loci developed for *Sheathia americana* and *S. grandis* that amplified for each species: *S. americana*, *S. grandis*, and *S. involuta*.

	S. americana	***S. americana*, site Sa-M1**	***S. grandis***	***S. involuta***	***S. involuta*, site Si-U22**
S. americana loci	Sam_02	Sam_02	Sam_02	Sam_02	Sam_02
	Sam_10	Sam_10	Sam_10	–	Sam_10
	Sam_08	Sam_08	Sam_08	–	–
	Sam_11	Sam_11	–	–	–
	Sam_01	Sam_01	–	–	–
	Sam_16	–	–	–	–
	Sam_19	–	–	–	–
	Sam_13	Sam_13	–	–	–
S. grandis loci	Sgr_21	Sgr_21	Sgr_21	Sgr_21	Sgr_21
	–	–	Sgr_04	Sgr_04	Sgr_04
	–	–	Sgr_19	–	Sgr_19
	–	–	Sgr_05	–	Sgr_05
	Sgr_17	–	Sgr_17	–	–
	–	–	Sgr_13	–	–
	–	–	Sgr_01	–	–
	Sgr_03	Sgr_03	–	–	–
Total number of loci per taxon	11	8	10	3	6

Note: There are two site-specific differences: (i) three loci that amplified for most *S. americana* gametophytes did not amplify for gametophytes from site Sa-M1, and (ii) several loci amplified for one *S. involuta* site, Si-U22, but did not amplify for the other *S. involuta* sites.

following the manufacturer's protocol, except for the lysis step in which the lysate was incubated at room temperature for 1 h, and DNA was eluted in either 200 μL (for gametophytes used for initial locus screening) or 100 μL (for gametophytes used for population genetic analyses) of molecular-grade water.

Sanger sequencing

We amplified and sequenced a 1282 bp portion of the *rbc*L gene to confirm species identification following methods from Vis et al. (2024). These sequences are available from GenBank (Benson et al., 2016; Table 1; see also Vis et al., 2024).

Microsatellite locus development

To develop microsatellite loci, we sent several *Sheathia americana* and *S. grandis* gametophytes to Microsynth ECOGENICS GmbH (Balgach, Switzerland) to generate single sequence repeat (SSR)-enriched sequences (Table S2). Di-, tri-, and tetra-nucleotide motifs were identified for each species, and primer sequences were designed using MSATCOMMANDER v1.0.8 (Faircloth, 2008). A set of quality filters was applied using R ver. 2022.07.2 (R Core Team, 2022) following a protocol modified from Schoebel et al. (2013; see also Crowell, Shainker-Connelly, Vis, & Krueger-Hadfield, 2024; Heiser et al., 2023; Ryan et al., 2021). Loci were removed if they had primer pairs that possessed a high pair penalty or if melting temperatures were too dissimilar. We also conducted a BLAST search in Geneious Primer v.2022.2.2 (Biomatters, Ltd., Auckland, New Zealand) 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 primer sequences. After this filtering step, 20 primer pairs remained for *S. americana*-generated loci, and 22 primer pairs remained for *S. grandis*-generated loci.

The 20 *Sheathia americana* and 22 *S. grandis* candidate loci were tested for cross-amplification using a panel of seven gametophytes, including *S. americana*, *S. grandis*, *S. involuta*, and a negative control (Table S2). We tested *S. americana*-generated loci across five *S. americana* gametophytes, one *S. grandis* gametophyte, and one *S. involuta* gametophyte. We tested *S. grandis*-generated loci across five *S. grandis* gametophytes, one *S. americana* gametophyte, and one *S. involuta* gametophyte. Simplex PCRs were performed to amplify each locus with a final volume of 20 μL: 2 μL DNA template, 1X Dreamtaq buffer (ThermoFisher Scientific, Cat. No. B65), 250 μM of each dNTP (Promega, Cat. No. R0192), 0.2 mg · mL^{-1} bovine serum albumin (BSA), 1 U Dreamtaq polymerase

(ThermoFisher Scientific, Cat. No. EP0701), and 250 nM of each oligo. The following program was used: 2 min at 95°C, followed by 35 cycles of 95°C for 30 s, 59°C for 30 s, and 72°C for 30 s, with a final extension of 5 min at 72°C. Amplification was confirmed by visually inspecting bands for the expected size on 1.5% agarose gels stained with GelRed (Biotium, Fremont, CA, United States, Cat. No. 41002-1).

Fragment analysis

Simplex PCRs were performed to amplify each locus (Table 3) using the same protocols described above for testing loci. We diluted 1.5 μL PCR product in 9.7 μL HiDi formamide (Applied Biosystems, Cat. No. 4311320) and 0.30 μL GS 500 LIZ (Applied Biosystems, Cat. No. 4322682) for fragment analysis at the University of Alabama at Birmingham Heflin Center for Genomic Sciences (Birmingham, Alabama, United States). Alleles were scored manually and binned using TANDEM (Matschiner & Salzburger, 2009) to ensure that all loci had an average rounding error below the recommended error threshold for each locus. We created a scoring guide which we used to determine each allele (Table S3).

Genetic and genotypic diversity

If there was no amplification at multiple loci after several PCR attempts, gametophytes were excluded from subsequent analyses. We considered a null allele as any gametophyte for which we did not obtain an allele at a given locus after discounting technical errors (see Krueger-Hadfield et al., 2011, 2013). We then calculated the following summary statistics to describe the reproductive system of sites with $n \geq 5$ gametophytes, to balance the recommendations and methods implemented in Krueger-Hadfield et al. (2021) and Stoeckel et al. (2021) with the realistic sample sizes that we were able to obtain at sites in which we could sample *Sheathia* gametophytes. Sites Sg-M14, Sg-U16, Si-D18, Si-U19, and Si-D20 had fewer than five genotyped gametophytes and were thus excluded from the following analyses. We calculated the unbiased probability of identity (*pid*) between sibs corrected for sample size to assess whether loci were of sufficient resolution to distinguish among individuals (Jacquard, 2012; Waits et al., 2001).

Next, we computed genotypic richness (*R*), which provides information on the relative proportion of repeated multilocus genotypes (MLGs), as: $R = \frac{(G-1)}{(N-1)}$, where *G* is the number of distinct genotypes (i.e., genets) and *N* is the number of genotyped gametophytes (Dorken & Eckert, 2001). We also calculated genotypic evenness (*D**; see box 3 in Arnaud-Haond et al., 2007), which provides information about the relative abundance of each MLG at a site. For genotypic evenness, if a site is dominated by a few dominant clones, *D** approaches 0. In contrast, if each genet is represented by an equal number of ramets at a site, *D** will approach 1 even if there are repeated MLGs. To facilitate interpretation, we plotted genotypic evenness (*D**) versus genotypic richness (*R*) following Baums et al. (2006) and Krueger-Hadfield et al. (2021). The plot allowed us to visualize reproductive system variation and to group sites based on low, intermediate, and high levels of uniparental reproduction. We have referred here to uniparental reproduction, as we were not able to distinguish between intragametophytic selfing and monospore production (see discussion in Shainker-Connelly et al., 2024). For neutral loci, genotypic evenness is expected to increase with increasing *R* if populations are maintained by sexual reproduction.

We calculated Pareto β, which describes the distribution of clonal membership. As *Sheathia* spp. gametophytes can be monoicous (Krueger-Hadfield et al., 2024), intragametophytic selfing results in similar population genetic patterns as clonality, so Pareto β cannot be used to disentangle the effects of clonal monospore production and intragametophytic selfing (see also Shainker-Connelly et al., 2024). Krueger-Hadfield et al. (2021) suggested that Pareto $\beta > 2$ was associated with low rates of clonality (and therefore, more sexual reproduction), $0.7 <$ Pareto $\beta < 2$ was associated with intermediate clonal rates, and Pareto $\beta < 0.7$ was associated with high clonal rates. These thresholds were meant to be used as suggested guidelines for grouping populations based on the rate of clonality (Krueger-Hadfield et al., 2021; Stoeckel et al., 2021). In our study, we used these ranges but considered uniparental reproduction, including intra- and intergametophytic selfing in addition to monospore production for *S. grandis, S. involuta*, and *S. americana.* (See Results for monoicy in *S. americana*.)

We calculated pairwise genetic distances for all gametophytes using GenAPoPop (Stoeckel et al., 2024) adapted for haploid data. For each site, we calculated linkage disequilibrium ($\bar{r}_d$) following Agapow and Burt (2001). To calculate pairwise linkage disequilibrium (LD) between loci within each site, we used Genepop ver.1.1.7 (Rousset, 2008) in R ver. 2022.07.2 (R Core Team, 2022). In partially clonal taxa, both clonality and selfing can lead to an increase in $\bar{r}_d$ and LD values and variance of these values within a species (Krueger-Hadfield et al., 2021). We calculated expected heterozygosity (H_E) following Stoeckel et al. (2021).

Genetic structure

To assess relationships of multilocus genotypes among sites and within and among regions, we used discriminant analysis of principal components (DAPC; Jombart et al., 2010) as implemented in the R package

TABLE 3 Summary statistics calculated for sites in which *Sheathia* sp. gametophytes were sampled, with site indicated (see Table 1).

Site	Species	*n*	*G*	*pid*	*R*	*D**	H_E	Pareto β	$\bar{r}_d$	Number of genotyped loci	Number of fixed loci
Sa-M1	*S. americana*	9	1	1	0	0	0	0.06	1	8[a]	8
Sa-U2	*S. americana*	26	26	6.72E-08	1	1	0.484	3.75	0.01	11	2
Sa-U3	*S. americana*	21	19	2.87E-04	0.90	0.99	0.235	2.39	0.02	11	5
Sa-U4	*S. americana*	19	17	1.60E-09	0.89	0.99	0.475	2.25	0.06	11	2
Sa-M5	*S. americana*	5	5	1.69E-06	1	1	0.443	1.58	0.01	11	2
Sg-M6	*S. grandis*	13	4	0.03	0.25	0.62	0.142	0.24	0.52	10	6
Sg-M7	*S. grandis*	16	4	0.02	0.20	0.65	0.183	0.07	0.34	10	5
Sg-M8	*S. grandis*	5	1	1	0	0	0	0.16	1	10	10
Sg-M9	*S. grandis*	24	3	0.20	0.09	0.47	0.063	0.18	0.42	10	8
Sg-M10	*S. grandis*	5	2	0.13	0.25	0.60	0.096	1.26	1	10	8
Sg-M11	*S. grandis*	15	5	0.01	0.29	0.73	0.181	0.37	0.37	10	6
Sg-D12	*S. grandis*	7	4	3.71E-03	0.50	0.71	0.192	0.40	0.20	10	3
Sg-D13	*S. grandis*	7	7	1.27E-03	1	1	0.273	2	−0.03	10	4
Sg-U15	*S. grandis*	12	1	1	0	0	0	0.04	1	10	10
Si-D17	*S. involuta*	10	3	0.04[b]	0.22	0.73	0.300	3.19	0.07	3	0
Si-U21	*S. involuta*	12	8	0.16[b]	0.64	0.89	0.468	0.79	−0.02	3	1
Si-U22	*S. involuta*	25	2	0.40	0.04	0.52	0.083	0.02	1	6[c]	5

Note: Summary statistics include the following: *n*, total number of gametophytes genotyped; *G*, number of unique genotypes; unbiased *pid*, probability of identity of sibs; *R*, genotypic richness; *D**, genotypic evenness; Pareto β, distribution of clonal membership, H_E, expected heterozygosity $\bar{r}_d$, multilocus estimate of linkage disequilibrium; and the number of fixed loci per site. *S. grandis* sites 14 and 16 and *S. involuta* sites 18, 19, and 20 were excluded from this table since an insufficient number of gametophytes were genotyped to calculate summary statistics (see Krueger-Hadfield et al., 2021). Only sites with enough genotyped gametophytes ($n \geq 5$) were included in these calculations.

[a]Loci Sam_16, Sam_19, and Sgr_17 did not amplify for individuals from this site but amplified for all other *S. americana* sites.

[b]Sites Si-D17 and Si-U21 were genotyped with three loci. The raw *pid* values were used because the corrected *pid* values require at least four loci.

[c]In addition to the three loci that amplified for all *S. involuta* sites, the loci Sam_10, Sgr_19, and Sgr_05 only amplified for Si-U22 and not for other *S. involuta* sites.

adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011). This multivariate analysis avoids making strong assumptions of the underlying genetic model. The method finds principal components (PCs) that best summarize the differences among a priori groups, while minimizing variation within groups (Jombart et al., 2009, 2010). To assess (i) relationships among all *Sheathia americana* sites, (ii) relationships among *S. americana* sites in New England, (iii) relationships among *S. grandis* regions, and then (iv) *S. grandis* sites within the state of Michigan, we performed four separate DAPC analyses. We did not perform a DAPC analysis on *S. involuta* because few loci were genotyped for this species. To determine the minimal number of PCs needed to correctly assess the proportion of group assignment for each replicate, we used the implemented cross-validation approach (xvalDapc). Discriminant analyses of principal components were performed with increasing numbers of PCs on 90% of our data, which were used to predict the proportion of group assignment on the remaining 10% of samples to identify the optimal number of PCs to retain. We repeated this approach 30 times for each species. Based on this cross-validation with the xvalDapc function, we retained the following number of PCs for each analysis: 26 PCs among all five *S. americana* sites (Figure S1), three PCs among the four New England *S. americana* sites (Figure S2), 19 PCs among three *S. grandis* regions (Figure S3), and nine PCs among the six *S. grandis* sites within Michigan (Figure S4). We used the find.clusters function to explore the relationship between the number of clusters (*K*) and the Bayesian information criterion (BIC; Figures S1–S4). To determine the drainage basins to which each site included in the DAPCs belonged, we overlaid shapefiles of level HUC-4 drainage basins (United States Geological Survey, 2024) with the GPS coordinates of each site over the Esri National Geographic basemap (Esri, 2011) in qGIS (version 3.34, 2024). The HUC-4 level was chosen to strike a balance between achieving realistic dispersal distances for this organism while distinguishing sites at the scale we sampled.

Data visualization

Figures were prepared using the following R packages: ggplot2 (Wickham, 2016), gridExtra (Auguie, 2017), Rcpp

(Eddelbuettel, 2013; Eddelbuettel & Balamuta, 2018; Eddelbuettel & Francois, 2011), sf (Pebesma, 2018), rnaturalearth (Massicotte & South, 2023), rnaturalearthdata, ggspatial (Dunnington, 2023), ggsn (Santos Baquero, 2019), pastecs (Grosjean & Ibanez, 2018), car (Fox & Weisberg, 2019), hierfstat (Goudet & Jombart, 2023), pegas (Paradis, 2010), adegenet (Jombart, 2008; Jombart & Ahmed, 2011), and poppr (ver. 2.9.4; Kamvar et al., 2014).

RESULTS

Characterization of microsatellite loci

A total of 16 loci were developed: eight from the *Sheathia americana* library and eight from the *S. grandis* library (Tables 2 and S4, Appendix S1). Multiple loci amplified for more than one species, resulting in 11 loci for *S. americana* (except for site Sa-M1, for which only eight of the 11 loci amplified), 10 loci for *S. grandis*, and three loci for *S. involuta* (except for site Si-U22, for which six loci amplified; Tables 2 and S5; Appendix S1).

We genotyped a total of 133 *Sheathia americana* gametophytes from five sites (average $n = 16$ gametophytes per site), 115 *S. grandis* gametophytes from 11 sites (average $n = 12$ gametophytes per site), and 86 *S. involuta* gametophytes from seven sites (average $n = 16$ gametophytes per site). Null alleles were not a concern for assessing patterns with these loci, as their frequency was less than a maximum of ~15% and often 0% (Table S6 and discussed in more detail in Appendix S1).

The unbiased probability of identity between pairs of gametophytes (*pid*) was 1.02×10^{-5} over the 71 *Sheathia americana* gametophytes genotyped with 11 loci (at sites Sa-U2, Sa-U3, Sa-U4, and Sa-M5) and 1 over the nine *S. americana* gametophytes genotyped with eight loci (at site Sa-M1). The *pid* was 0.008 over the 104 *Sheathia grandis* gametophytes genotyped with 10 loci at the following sites: Sg-M6, Sg-M7, Sg-M8, Sg-M9, Sg-M10, Sg-M11, Sg-D12, Sg-D13, and Sg-U15. The *pid* was 0.309 over the 56 *Sheathia involuta* gametophytes genotyped with three loci (Si-D17, Si-U21, and Si-U22), and 0.40 over the 25 *S. involuta* gametophytes genotyped with six loci (site Si-U22; Table 3).

Sexual system variation

We determined that two *Sheathia americana* sites, Sa-M1 and Sa-M5, were monoicous, with carpogonia and spermatangia present on the same thallus (Tables 1 and S7). Only female gametophytes were observed from the three remaining sites, so their sexual systems were designated as undetermined. Three *Sheathia involuta* sites, Si-D17 ($n = 16$), Si-D18 ($n = 4$), and Si-D20 ($n = 8$), were dioicous because they had a mix of thalli that had only carpogonia and those that had only spermatangia (Tables 1 and S7). Three sites, Si-U19 ($n = 1$), Si-U21 ($n = 1$), and Si-U22 ($n = 1$), were classified with undetermined sexual systems because each had only one thallus with only female reproductive structures. We determined the sexual system for nine of the 11 *Sheathia grandis* sites. Seven sites, Sg-M6 ($n = 16$), Sg-M7 ($n = 10$), Sg-M8 ($n = 6$), Sg-M9 ($n = 4$), Sg-M10 ($n = 4$), Sg-M11 ($n = 16$), and Sg-M14 ($n = 1$) were classified as monoicous, with thalli that had both spermatangia and carpogonia (Tables 1 and S7). Two sites, Sg-D12 ($n = 2$) and Sg-D13 ($n = 2$), were classified as dioicous, with thalli that had only carpogonia or only spermatangia. The remaining sites were classified as having undetermined sexual systems because either each had thalli with only carpogonia and no spermatangia or we did not have physical specimens to examine (Table S7).

Genotypic diversity

Genotypic richness (R) values ranged from 0 to 1 for *Sheathia americana* and *S. grandis* and from 0.04 to 0.64 for *S. involuta* (Table 3; Figure 2). Most *S. americana* sites had high genotypic richness in which repeated MLGs were always distributed within but not among sites (Table 3; Figure 2); however, at site Sa-M1 ($n = 9$), all genotyped gametophytes belonged to the same MLG (Table 3). Most *S. grandis* sites had low to intermediate genotypic richness ($R \leq 0.50$), except for Sg-D13, which had an R of 1 (Table 3; Figure 2). Most repeated *S. grandis* MLGs were reencountered within and not among sites, but one MLG was repeated across sites Sg-M6 ($n = 8$ gametophytes), Sg-M8 ($n = 5$ gametophytes), and Sg-M11 ($n = 7$ gametophytes; Table S8a). Several *S. involuta* MLGs were repeated among multiple sites (Table S8b). This pattern may have been due to low resolution of genotypes as indicated by the high *pid* value for *S. involuta*.

Genotypic evenness (D^*) was close to 1 at most *Sheathia americana* sites, except at Sa-M1 where D^* was zero (Table 3; Figures 2 and 3). Among *S. grandis* sites, D^* varied from 0 to 1, with most values greater than 0.50 (Table 3, Figure 2). Among *S. involuta* sites, D^* ranged from 0.52 to 0.89 (Table 3, Figure 2).

By visualizing the relationship between D^* and R, we identified three categories based on their distributions on the plot: high uniparental reproduction ($R < 0.30$), intermediate uniparental reproduction ($0.30 < R < 0.75$), and low uniparental reproduction ($R > 0.75$; Figure S5). All *Sheathia americana* sites had high or low uniparental reproduction, and *S. grandis* and *S. involuta* sites were distributed among all three categories.

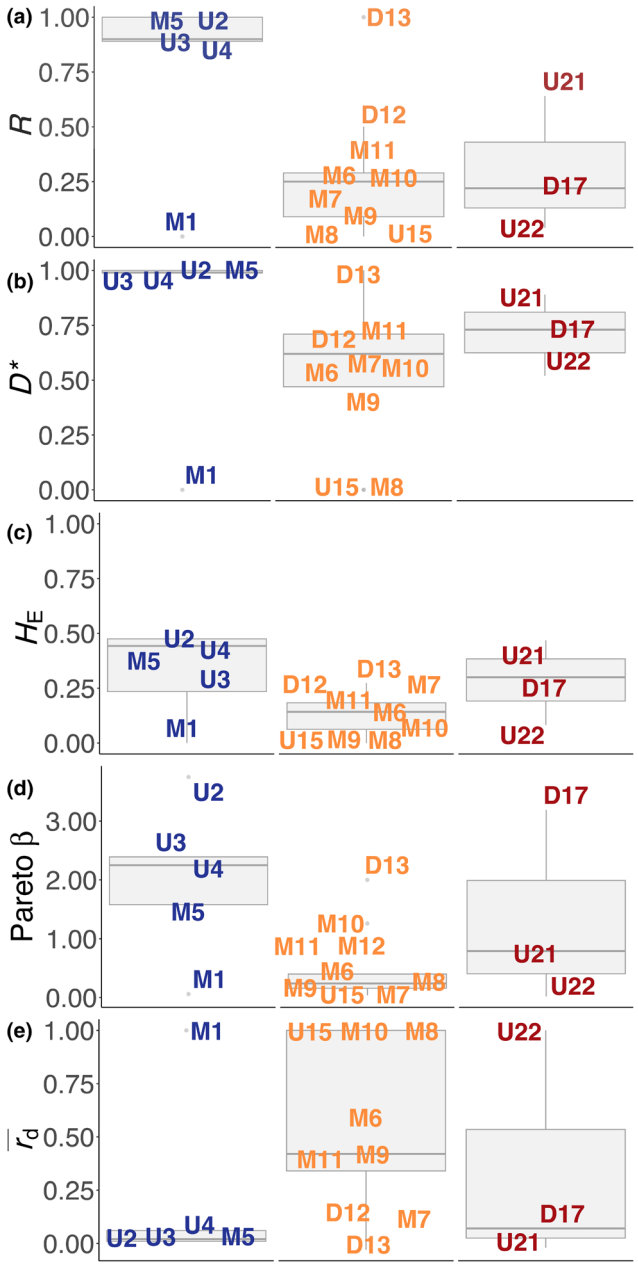

FIGURE 2 Boxplots of (a) genotypic richness (*R*), (b) genotypic evenness (*D**), (c) Pareto β, (d) linkage disequilibrium ($\bar{r}_d$), and (e) expected heterozygosity (H_E) values per site in *Sheathia americana* (blue), *S. grandis* (yellow), and *S. involuta* (red). Boxes represent the interquartile range, the middle lines are medians, whiskers represent the 1.5 interquartile ranges, and the gray dots are outliers. The letter preceding the site number indicates the sexual system: D, "dioicous"; M, "monoicous"; U, "unknown". *S. grandis* sites 14 and 16 and *S. involuta* sites 18, 19, and 20 were excluded from these plots due to an insufficient number of genotyped gametophytes. See Table 1 for site numbers and the number of microsatellite loci used for calculating summary statistics per site.

For *Sheathia americana*, Pareto β values were variable across categories of low (Pareto β > 2), intermediate (0.7 < Pareto β < 2), and high clonal rates (Pareto β < 0.7; see Krueger-Hadfield et al., 2021), ranging from 0.06 (Sa-M1) to 3.75 (Sa-U2; Table 3, Figure 2). For *S. grandis*, Pareto β values ranged from 0.04 (Sg-U15) to 2, and most sites were in the high (Pareto β < 0.7) or intermediate (0.7 < Pareto β < 2) categories of clonal rates (Sg-D13; Table 3, Figure 2). For *S. involuta*, Pareto β values were variable and ranged from 0.02 (Si-U22) to 3.19 (Si-D17), representing all three categories of clonal rates (Table 3, Figure 2).

Genetic diversity

Expected heterozygosity (H_E) was low to intermediate for all *Sheathia* sites, regardless of species. Expected heterozygosity ranged from 0 (Sa-M1) to 0.484 (Sa-U2) across *S. americana* sites, 0 (sites Sg-M8 and Sg-U15) to 0.273 (Sg-D13) among *S. grandis* sites, and 0.083 (Si-U22) to 0.468 (Si-U21) among *S. involuta* sites (Table 3, Figure 2).

Most *Sheathia* sites had low linkage disequilibrium values ($\bar{r}_d$). Among *S. americana* sites, linkage disequilibrium ($\bar{r}_d$) values ranged from 0.01 (sites Sa-U2 and Sa-M5) to 1 (site Sa-M1; Table 3, Figure 2). All sites except Sa-M1 had values ≤0.06. Among *S. grandis* sites, linkage disequilibrium ($\bar{r}_d$) values ranged from −0.03 (site Sg-D13) to 1 (sites Sg-M8, Sg-M10, and Sg-U15; Table 3, Figure 2). Most sites had values <0.50. Among *S. involuta* sites, linkage disequilibrium ($\bar{r}_d$) values ranged from −0.02 (Si-U21) to 1 (Si-U22; Table 3, Figure 2). Sites Si-U21 and Si-D17 had low values, close to zero. Pairwise LD between loci could not be tested for most locus pairs of all three species because within sites there was little to no variation in the alleles at a locus. For those pairs that could be tested, the majority had non-significant LD (Table S9).

The maximum possible number of diverging alleles between a pair of *Sheathia americana* gametophytes was 11 because this was the number of loci used for all sites except Sa-M1, for which the maximum possible number of diverging alleles was eight. The maximum number of diverging alleles observed between any pair of *Sheathia americana* gametophytes was nine (Sa-U4, Sa-U2, Sa-M5). At site Sa-M1, there were no pairs of diverging alleles, meaning that only one multilocus genotype was sampled. The maximum possible number of diverging alleles between a pair of *S. grandis* gametophytes was 10, as 10 loci were used. The maximum number of diverging alleles observed between any pair of *S. grandis* gametophytes was six (Sg-D12). At Sg-U15 and Sg-M8, there were no pairs of diverging alleles, meaning that only one multilocus genotype was sampled at each of those sites. The maximum possible number of diverging alleles between a pair of *S. involuta* gametophytes was three, except for site Si-U22, for which the maximum possible number of diverging alleles was six because six loci were used. The maximum number of diverging alleles observed between

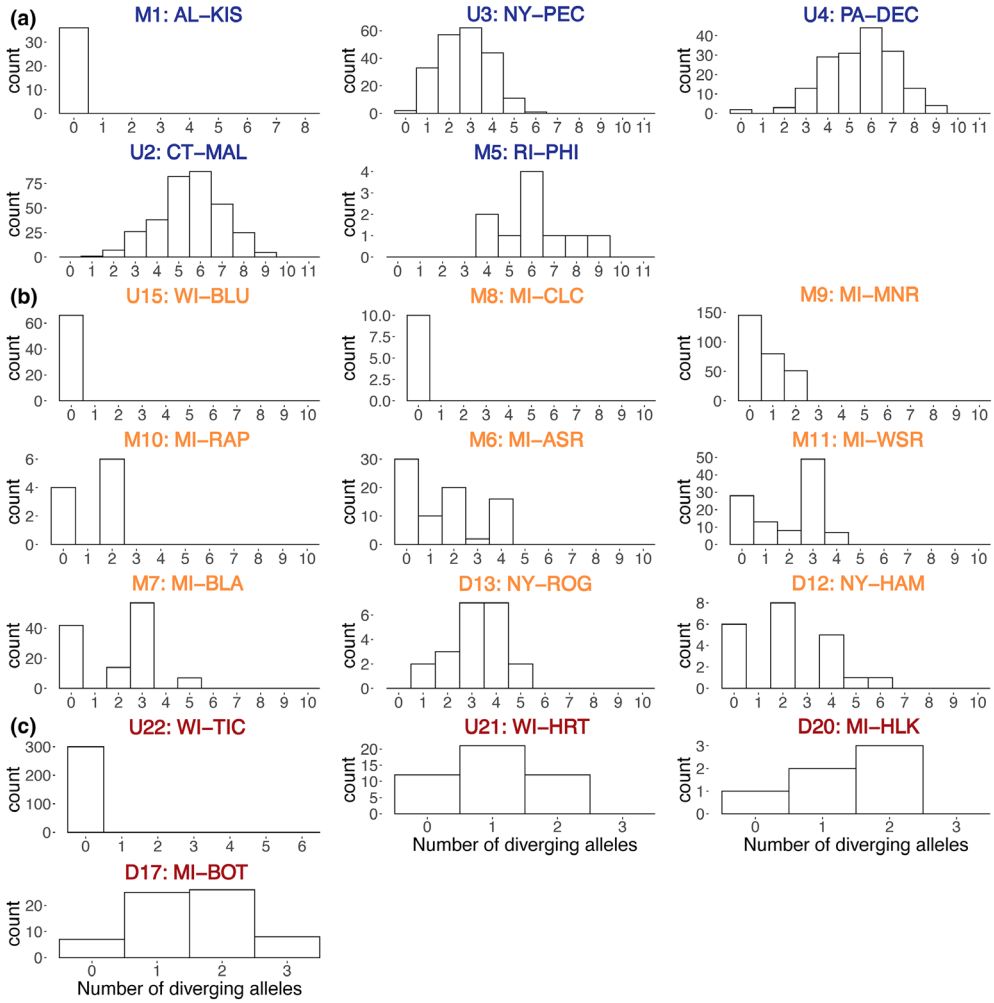

FIGURE 3 The distribution of counts of diverging alleles per site for (a) *Sheathia americana* (blue), (b) *S. grandis* (orange), and (c) *S. involuta* (red), arranged by increasing numbers of diverging alleles for each species. The *x*-axis represents the number of diverging alleles between each pair of gametophytes from the same site, with the range indicating the number of genotyped loci. The *y*-axis ("count") represents the number of pairs of gametophytes with the given number of diverging alleles. Site numbers and abbreviations are shown in Table 1. The letter preceding the site name indicates the sexual system: D, "dioicous"; M, "monoicous"; U, "unknown".

any pair of *S. involuta* gametophytes was three (Si-D17). Site Si-U22 had zero pairs of diverging alleles between gametophytes, meaning that only one multilocus genotype was sampled at this site (Figure 3).

Genetic structure

We observed strong genetic structure for both *Sheathia americana* and *S. grandis*, the two species for which the DAPC was possible. For all *S. americana* sites, there was a continuous decline in the BIC as the number of clusters (*K*) increased (Figure S1c), suggesting a stepping-stone pattern of dispersal. The first principal component axis 1 (PC1) explained 45.0%, PC2 explained 26.8%, and PC3 explained 23.6% of the genetic variation. Site Sa-M1 in northern Alabama was separated from the remaining four sites, which are all found in the northeastern United States (Figure 4a,b). Along the first two PC axes, two sites sharing the Coastal Connecticut HUC-4 drainage basin clustered together (sites Sa-U2 and Sa-M5; Figure 4b). The remaining sites were genetically distinct and were not from the same drainage basin. This pattern did not meaningfully change when we removed the very genetically distinct site Sa-M1 from Alabama (Figure S2).

For *Sheathia grandis*, we observed a similar pattern between the relationship of BIC and *K*, partitioning regions and sites (Figures S3 and S4). Each region (MI, WI, and NY) was separated along the first two PC axes, and sites within regions clustered together (Figure 4c). Within Michigan (MI), sites Sg-M11 (MI-WSR), Sg-M10 (MI-RAP), Sg-M6 (MI-ASR), and Sg-M8 (MI-CLC) clustered together, and sites Sg-M9 (MI-MNR) and Sg-M7 (MI-BLA) were further separated from the other sites (Figure 4d). The first two PCs explained 100% of the variation, with PC1 explaining 70.7% and PC2

explaining 25.9% (Figure 4d). Each site in Michigan was in a different HUC-4 drainage basin.

DISCUSSION

We characterized the sexual systems and reproductive modes within five *Sheathia americana* sites, nine *S. grandis* sites, and three *S. involuta* sites using microsatellite loci that we developed for this study. We also used genetic diversity to study genetic differentiation among these sites across eastern United States. We detected sexual-system variation among and within species. *Sheathia americana* exhibited greater genotypic diversity and had generally greater Pareto β values when compared with *S. grandis* and *S. involuta*. However, contrary to our knowledge before this study (Salomaki et al., 2014; Vis & Necchi, 2021), we discovered two monoicous *S. americana* populations, suggesting greater sexual-system variation in this taxon than expected. *Sheathia americana* may be reproducing sexually with lower rates of selfing at the three sites for which we could not determine the sexual system, hinting that they may be dioicous. *Sheathia involuta* only had three sites for which we could calculate summary statistics, and we generally calculated lower *pid* values, suggesting limitations in our ability to determine the prevailing reproductive mode. In *S. grandis*, we had a more robust comparison of monoicous versus dioicous sites, and there was a large amount of variation in the reproductive systems as we predicted would occur due to the sexual-system variation in this species. Further, the DAPCs we generated showed evidence of genetic structure that likely represents vicariant events during the last glacial maximum (see also Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024 in

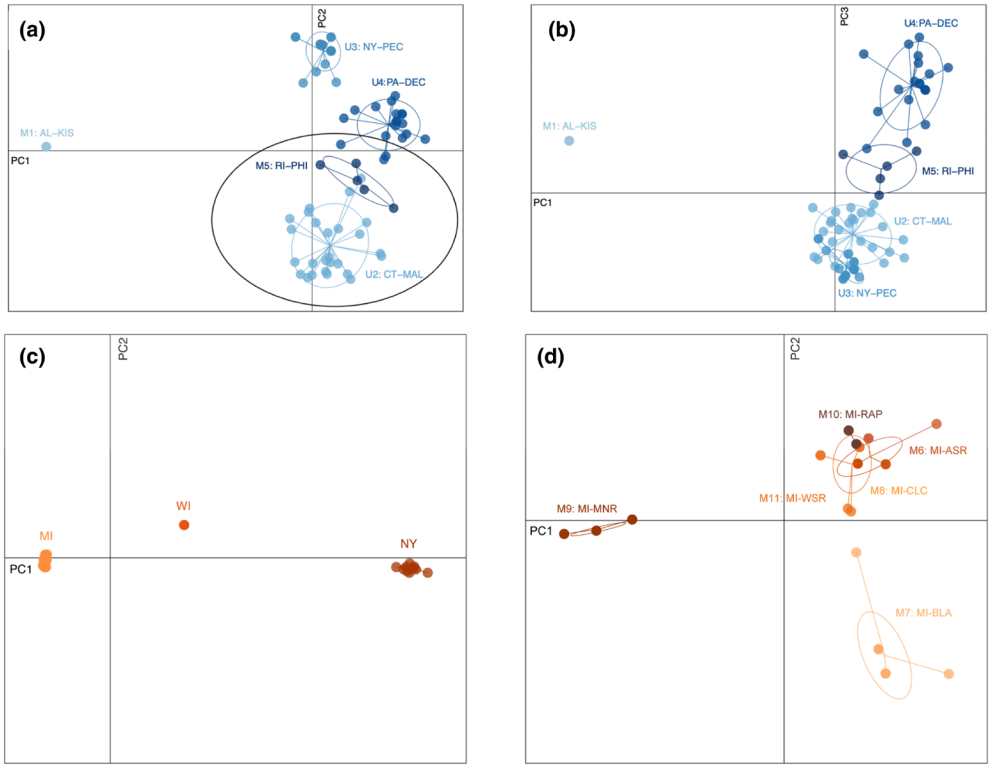

FIGURE 4 (a) Discriminant analysis of principal components (DAPC) of *Sheathia americana* multilocus genotypes (MLGs) of gametophytes collected from sites in Alabama and the northeast United States (Figure 1, Table 1). Points represent gametophytes and colors correspond with the five a priori groups determined by site. The first two principal components (PCs) are shown. The amount of variation explained by each PC is as follows: PC1 (45.0%) and PC2 (26.8%). The black ellipse indicates the two sites that share a HUC-4 drainage basin (Coastal Connecticut). (b) DAPC of the same *S. americana* multilocus genotypes (MLGs) in (a), showing the first and third PC. The amount of variation explained by each PC is as follows: PC1 (45.0%) and PC3 (23.6%). Site numbers and abbreviations are shown in Table 1. The letter preceding the site name indicates the sexual system: D, "dioicous"; M, "monoicous"; U, "unknown". (c) Discriminant analysis of principal components (DAPC) of *Sheathia grandis* multilocus genotypes (MLGs) among the three states in which gametophytes from this species were collected (Figure 1, Table 1). Points represent gametophytes and colors correspond with the three a priori groups determined by region. The first two principal components (PCs) are shown, and the amount of variation explained by each PC is as follows: PC1 (99.1%) and PC2 (0.9%). (d) Discriminant analysis of principal components (DAPC) of *Sheathia grandis* multilocus genotypes (MLGs) of gametophytes collected from sites in Michigan (Figure 1, Table 1). Points represent gametophytes and colors correspond with the six a priori groups determined by site. The first two principal components (PCs) are shown, and the amount of variation explained by each PC is as follows: PC1 (70.7%) and PC2 (25.9%). Site numbers and abbreviations are shown in Table 1. The letter preceding the site name indicates the sexual system: D, "dioicous"; M, "monoicous"; U, "unknown".

Batrachospermum gelatinosum) and patterns of limited contemporary gene flow. There are likely occasional occurrences of long-distance dispersal via water flow and/or zoochory based on shared genetic ancestries as visualized using DAPC. Below, we discuss the population genetic patterns for *Sheathia* spp. and their implications for the evolution of sexual and reproductive system variation in batrachospermalean algae, haploid-diploid organisms, and eukaryotes more generally.

The influence of the sexual system on reproductive system variation

Dioicy was previously considered a characteristic trait of the species *Sheathia americana* (Salomaki et al., 2014; Vis & Necchi, 2021), but we established that two sites, Sa-M1 (Alabama) and Sa-M5 (Rhode Island), were monoicous. We were unable to characterize the sexual systems of the remaining three sites, but our results suggest that the sexual system of *Sheathia* spp. is more labile than previously thought. Monoicy and dioicy are not always reliable as taxonomic traits (Entwisle et al., 2004), and it appears we cannot use this trait as a taxonomic characteristic for *Sheathia* spp. Instead, the sexual system should be assessed for each site at the time of sampling. Nevertheless, as predicted, *S. americana* had higher genetic and genotypic diversity overall compared with *S. grandis* and *S. involuta*. Four of the five *S. americana* sites, excluding Sa-M1, likely involve more sexual reproduction, with less selfing and/or monospore production. Site Sa-M1and Sa-M5 had Pareto β values less than 2, which Krueger-Hadfield et al. (2021) used as a cut-off for lower rates of clonality. However, we are not able to disentangle between intragametophytic selfing and monospore production in monoicous populations (Shainker-Connelly et al., 2024, 2025). Intragametophytic selfing results in instantaneous, genome-wide homozygosity, rendering all chantransia produced from those gamete unions genotypically identical. All subsequent gametophytes produced from a fully homozygous chantransia will also share the same genotype. Site Sa-M1 is at a much lower latitude than the presumed range for *S. americana*, previously thought to be limited to New England and Newfoundland, Canada (Salomaki et al., 2014; Vis & Necchi, 2021). Such a range extension may be facilitated by monoicy (i.e., Baker's Law, Baker, 1955; Pannell et al., 2015). For example, in the aquatic plant *Sagittaria latifolia*, monoecious populations occurred more frequently in ephemeral habitats; dioecious populations were more common in stable wetlands (Yakimowski & Barrett, 2014). Our observations raise questions about *S. americana* and its range. Future studies should examine whether monoicous sites of *S. americana* and other *Sheathia* spp. may inhabit more marginal environments or geographical range edges compared to dioicous sites. Seasonal sampling, too, could better resolve temporal variation in sex allocation in monoicous populations. Moreover, at three of our five sites, we were unable to determine the sexual system, though our genetic data hinted at dioicy. Characterization of gametophytes as monoicous or dioicous is critical for interpreting population genetic data correctly, as demonstrated by our observation that *S. americana* exhibits greater sexual-system variation than previously described in the literature (Salomaki et al., 2014; Vis & Necchi, 2021).

We had predicted greater variation in the prevailing reproductive mode in *Sheathia involuta* and *S. grandis* because both species have been documented as having monoicous and dioicous gametophytes. However, *S. involuta* and *S. americana* displayed similar variation for some summary statistics (e.g., Pareto β). Moreover, with only five *S. americana* sites (three of which were underdetermined) and three *S. involuta* sites, it is difficult to accurately describe variation. *Sheathia grandis*, for which we have a much more robust sampling of sites and monoicous versus dioicous populations, displayed ample variation as assessed by genotypic and genetic diversity, partially supporting our hypothesis. Monoicous *S. grandis* sites tended to have lower genotypic richness (R) and evenness (D^*), genetic diversity (H_E), and Pareto β while exhibiting generally larger estimates of multilocus linkage disequilibrium ($\bar{r}_d$). In monoicous populations, there is likely a combination of sexual (intragametophytic selfing, intergametophytic selfing, and outcrossing) and asexual reproduction (monospore production) leading to variation among sites. For dioicous populations, intragametophytic selfing is not possible, but monospore production and intergametophytic selfing may both contribute to the patterns we observed. For the moment, we are unable to distinguish between these different reproductive modes.

The relationship between genotypic richness and evenness (Figure S5) for *Sheathia grandis* and *S. involuta* resembled that of another red macroalga in the Batrachospermales: *Batrachospermum gelatinosum* (Shainker-Connelly et al., 2024). As *Batrachospermum gelatinosum* is obligately monoicous, our results do not directly support the hypothesis that the sexual-system variation in the species *S. grandis* and *S. involuta* is the driving force for the reproductive-system variation observed in these taxa. For *S. grandis*, the characterization of the reproductive system based on genotypic richness and evenness (Figure 3) was similar to that indicated by Pareto β values. Summary statistics were less consistent for *S. involuta*, which may be an artifact of the lower number of sites sampled for this species. Future population genetic studies should test for correlations between the sexual system (i.e., monoicy and dioicy) and the reproductive system by assessing the sexual system of each site sampled.

Though we characterized gametophytes as monoicous or dioicous, it is possible that there is undetected trioicy (i.e., the presence of male, female, and hermaphroditic gametophytes at the same site). Although many genetic models have predicted that trioecy (in organisms in which sex is determined in the diploid phase) should not be maintained as a stable evolutionary state (Lande & Schemske, 1985), it has been observed in multiple taxa including crustaceans (Sassaman & Weeks, 1993), plants (Mirski et al., 2017), and nematodes (Chaudhuri et al., 2015; Kanzaki et al., 2017). Anderson et al. (2020) demonstrated that trioecy could provide "the best of both worlds" (p. 519) by increasing the likelihood of both outcrossing (to minimize inbreeding depression) and dispersal (in which only one hermaphroditic individual is required to found new populations). In future studies, inspecting a representative number of thalli from multiple sites will help to characterize the proportion of trioicous populations if they exist in the genus *Sheathia* and to test whether this is an evolutionary stable state in haploid-diploid organisms, including freshwater reds.

Genetic structure and connectivity

As expected, we observed strong patterns of genetic structure in *Sheathia americana* and *S. grandis*. These patterns matched some of those observed in another freshwater red alga, *Batrachospermum gelatinosum*, in which sites were not only separated by drainage basin but also were each genetically distinct at smaller spatial scales (Crowell, Shainker-Connelly, Krueger-Hadfield, & Vis, 2024). Although we did not have the same level of site replication as Crowell, Shainker-Connelly, Krueger-Hadfield, and Vis (2024) in each drainage basin, we observed geographic structure at large spatial scales for the two *Sheathia* spp. Unlike the *Batrachospermum gelatinosum* study, for *Sheathia americana*, two sites shared the same HUC-4 drainage basin (Coastal Connecticut), and we observed genetic clustering between these sites (Sa-M5: RI-PHI and Sa-U2: CT-MAL). These results may indicate that admixture was driven by drainage patterns and water flow rather than distances "as the crow flies" as expected in zoochorous dispersal. Carposporophytes can detach and travel 5–35 m downstream (Hambrook & Sheath, 1991), potentially driving dispersal and genetic connectivity within watersheds.

Each *Sheathia grandis* region (MI, WI, and NY) was genetically distinct. Within the state of Michigan, sites Sg-M9 (MI-MNR) and Sg-M7 (MI-BLA) were genetically distinct, but sites Sg-M6 (MI-ASR), Sg-M10 (MI-RAP), Sg-M8 (MI-CLC), and Sg-M11 (MI-WSR) clustered together. This pattern was not correlated with drainage basins or geographic distance but may have been driven by incidents of medium-to-long-distance dispersal. Hall and Vis (2002) and Chiasson et al. (2003) observed complex phylogeographic patterns in the freshwater red alga *Virescentia viride-americana* (as *Batrachospermum helminthosum*) that they hypothesized were driven by migrating birds as dispersal vectors. However, it is unknown whether freshwater red algal spores, carposporophytes, or gametophytic fragments have sufficient desiccation tolerance to survive transport. Overall, our results suggest that the genetic structure of *Sheathia* spp. is likely mainly driven by patterns of water flow, with occasional long-distance dispersal events facilitated by animals.

The sexual system may also influence genetic structure and connectivity. Vekemans and Hardy (2004) observed that angiosperms engaging more frequently in selfing as compared to outcrossing tended to have greater spatial genetic structure. However, there are exceptions to this pattern. For example, in the aquatic vascular plant *Sagittaria latifolia*, dioecious populations are more genetically structured than monoecious ones (Yakimowski & Barrett, 2014). More sites would be needed to test for the influence of the sexual system on genetic structure in *Sheathia* spp. and could be informed by measurements of ecological or morphological features.

Conclusions and future directions

The genus *Sheathia* provides an opportunity to study the evolution of reproductive system variation, particularly in relation to the haploid-diploid life cycle. We observed that the sexual system varies within and among *Sheathia* spp. *Sheathia americana* exhibited both monoicous and dioicous populations, raising questions about using the sexual system as a taxonomic tool in this genus. Except for at site Sa-M1, *S. americana* was likely engaging in greater outcrossing as compared to the two other *Sheathia* spp., even in the site that had monoicous gametophytes in Rhode Island (Sa-M5). We had the largest sample size for *Sheathia grandis* and observed wide variation in dioicy and monoicy in this species. The variation we observed across *S. grandis* using our available summary statistics supported our hypothesis of greater reproductive system variation within this species and corresponded with the patterns observed in angiosperms (e.g., Barrett, 2002). Future studies could expand this work by testing these microsatellite loci for cross-amplification in additional species, such as *S. confusa* or *S. heterocortica*, bypassing the laborious process of developing new loci and allowing characterization of reproductive systems more broadly across the genus.

Our study has additionally confirmed limitations discussed by Shainker-Connelly et al. (2024, 2025): We cannot easily disentangle the effects of sexual versus asexual reproduction due to the haploid-diploid life

cycle and the unique attributes of the batrachospermalean life cycle (e.g., microscopic diploid phase). In kelps (e.g., Billot et al., 2003), the diploid sporophytes enable the calculation of a whole range of summary statistics and the use of different analytical techniques. However, with the macroscopic life phase consisting of haploid gametophytes in Batrachospermalean red algae, we are, at present, limited in our ability to robustly describe the reproductive system. Yet, this study has provided a framework for future work to develop novel techniques for genotyping chantransia and to add more polymorphic markers to enhance the resolution of gametophytic genotypes. Overall, the data generated from this study and recommendations for future directions could improve our understanding of the evolution of sex by elucidating the relationship between sexual- and reproductive-system variation and population genetic structure in haploid-diploid taxa.

AUTHOR CONTRIBUTIONS

Sarah J. Shainker-Connelly: Conceptualization (equal); data curation (lead); formal analysis (equal); funding acquisition (supporting); investigation (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Solenn Stoeckel:** Formal analysis (equal); funding acquisition (supporting); software (lead); writing – original draft (supporting); writing – review and editing (equal). **Morgan L. Vis:** Conceptualization (equal); data curation (supporting); formal analysis (supporting); funding acquisition (supporting); investigation (supporting); resources (equal); supervision (supporting); writing – original draft (supporting); writing – review and editing (equal). **Stacy A. Krueger-Hadfield:** Conceptualization (equal); data curation (supporting); formal analysis (equal); funding acquisition (lead); investigation (equal); resources (equal); software (supporting); supervision (lead); visualization (equal); writing – original draft (equal); writing – review and editing (equal).

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DATA AVAILABILITY STATEMENT

Genotype files and markdown files for this study are available at Zenodo: Shainker-Connelly, S., Stoeckel, S., Vis, M. L., Krueger-Hadfield, S., & Krueger-Hadfield, S. (2025). Monoicy, dioicy, and genetic structure in three species of Sheathia (Batrachospermales, Rhodophyta) [Data set]. Zenodo. https://doi.org/10.5281/zenodo.15381199

ORCID

Solenn Stoeckel https://orcid.org/0000-0001-6064-5941
Morgan L. Vis https://orcid.org/0000-0003-3087-1563
Stacy A. Krueger-Hadfield https://orcid.org/0000-0002-7324-7448

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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. Supplementary results.

Figure S1. Discriminant analysis of principal components (DAPC) depicting relationships of multilocus genotypes (MLGs) of *Sheathia americana* within five sites in the eastern United States: (a) Using the compoplot function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011), we determined the a priori assignment of five streams. (b) Cross-validation using the xvalDapc function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011). The optimal number of principal components to retain was 26. (c) The plot of number of clusters (K) and the Bayesian Information Criterion (BIC) for the five *S. americana* sites.

Figure S2. Discriminant analysis of principal components (DAPC) depicting relationships of multilocus genotypes (MLGs) of *Sheathia americana* within four sites in New England: (a) Using the *compoplot* function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011), we determined the a priori assignment of five streams. (b) Cross-validation using the *xvalDapc* function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011). The optimal number of principal components to retain was 26. (c) The plot of number of clusters (K) and the Bayesian information criterion (BIC) for the four *S. americana* sites in New England.

Figure S3. Discriminant analysis of principal components (DAPC) depicting relationships of multilocus genotypes (MLGs) of *Sheathia grandis* within the states of Michigan, Wisconsin, and New York. (a) Using the *compoplot* function as implemented in the R package *adegenet* version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011), we determined the a priori assignment of three regions: the lower peninsula of Michigan (LP_MI), New York (NY), and Wisconsin (WI). (b) Cross-validation using the *xvalDapc* function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011). The optimal number of principal components to retain was 19. (c) The plot of number of clusters (K) and the Bayesian information criterion (BIC) for the three *S. grandis* regions.

Figure S4. Discriminant analysis of principal components (DAPC) depicting relationships of multilocus genotypes (MLGs) of *Sheathia grandis* within Michigan (MI). (a) Using the *compoplot* function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011) we determined the a priori assignment of the six *S. grandis* sites sampled in MI: Sg-M7 (MI-BLA), Sg-M11 (MI-WSR), Sg-M9 (MI-MNR), Sg-M8 (MI-CLC), Sg-M6 (MI-ASR), Sg-M10 (MI-RAP). (b) Cross-validation using the *xvalDapc* function as implemented in the R package adegenet version 2.1.10 (Jombart, 2008; Jombart & Ahmed, 2011). The optimal number of principal components to retain was nine. (c) The plot of number of clusters (K) and the Bayesian Information Criterion (BIC) for the six *S. grandis* sites in Michigan.

Figure S5. Reproductive mode variation shown as genotypic evenness (D^*, see box 3 from Arnaud-Haond et al., 2007) versus genotypic richness (R). Each number indicates a *Sheathia* sp. site (see Table 1). Dashes are used to indicate the location of some sites on the plot to avoid overlap. Sites are divided into those with high uniparental reproduction ($R < 0.40$), intermediate uniparental reproduction ($0.40 < R < 0.75$), and low uniparental reproduction ($R > 0.75$). The letter preceding the site name indicates the sexual system: M = "monoicous", D = "dioicous", and U = "unknown". The following abbreviations are used to indicate each species: Sa for *S. americana* (blue), Sg for *S. grandis* (orange), and Si for *S. involuta* (red). *S. grandis* sites 14 and 16 and *S. involuta* sites 18, 19, and 20 were excluded from these plots due to an insufficient number of genotyped gametophytes.

Table S1. Water clarity and color, and stream bed composition for each site.
Table S2. Sites from which Sheathia gametophytes were used in microsatellite development.
Table S3. Raw size ranges for each binned allele.
Table S4. Microsatellite locus information for *Sheathia* spp. Locus name, repeat motif, expected size, oligo sequences, agarose gel amplification profile, fluorochrome used on the forward oligo, and fragment analysis (FA) amplification profile.
Table S5. The number of alleles per locus for each Sheathia species.
Table S6. Null allele frequencies for each locus for (a) *Sheathia americana*, (b) *S. grandis*, and (c) *S. involuta* were determined by non-amplification after 2-3 PCR attempts.
Table S7. Information regarding the sexual system determined for each site.
Table S8. Names of multilocus genotypes (MLGs) repeated among sites for (a) *Sheathia grandis* and (b) the *S. involuta* sites genotyped with three loci.
Table S9. Linkage disequilibrium (LD) between pairs of loci was tested using Genepop ver.1.2.2 (Rousset, 2008) in R ver. 2022.07.2 (R Core Team, 2022).

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