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RESEARCH ARTICLE

Interpopulation variation in inbreeding is primarily driven by tolerance of mating with relatives in a spermcasting invertebrate

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Abstract

The degree to which individuals inbreed is a fundamental aspect of population biology shaped by both passive and active processes. Yet, the relative influences of random and non-random mating on the overall magnitude of inbreeding are not well characterized for many taxa. We quantified variation in inbreeding among qualitatively accessible and isolated populations of a sessile marine invertebrate (the colonial ascidian *Lissoclinum verrilli*) in which hermaphroditic colonies cast sperm into the water column for subsequent uptake and internal fertilization. We compared estimates of inbreeding to simulations predicting random mating within sites to evaluate if levels of inbreeding were (1) less than expected because of active attempts to limit inbreeding, (2) as predicted by genetic subdivision and passive inbreeding tolerance, or (3) greater than simulations due to active attempts to promote inbreeding via self-fertilization or a preference for related mates. We found evidence of restricted gene flow and significant differences in the genetic diversity of *L. verrilli* colonies among sites, indicating that on average colonies were weakly related in accessible locations, but their levels of relatedness matched that of first cousins or half-siblings on isolated substrates. Irrespective of population size, progeny arrays revealed variation in the magnitude of inbreeding across sites that tracked with the mean relatedness of conspecifics. Biparental reproduction was confirmed in most offspring (86%) and estimates of total inbreeding largely overlapped with simulations of random mating, suggesting that interpopulation variation in mother–offspring resemblance was primarily due to genetic subdivision and passive tolerance of related mates. Our results highlight the influence of demographic isolation on the genetic composition of populations, and support theory predicting that tolerance of biparental inbreeding, even when mates are closely related, may be favoured under a broad set of ecological and evolutionary conditions.

KEYWORDS
biparental inbreeding, inbreeding tolerance, marine invertebrate, philopatry

1 | INTRODUCTION

The extent to which populations are demographically open or closed strongly influences the structure of genetic variation, distribution of relatedness among individuals, and potential for inbreeding (Hixon et al., 2002; Pannell, 2015; Shields, 1982). Within the bounds set by demography, mating systems and levels of inbreeding are shaped by traits regulating the tendency to avoid, tolerate, or prefer related mates (Chesser & Ryman, 1986; Szulkin et al., 2013). Self-incompatibility, kin discrimination during mate choice, and gametic recognition systems that block fertilization among relatives (i.e. gametic allorecognition) limit the magnitude of inbreeding and are predicted to be common when the costs of inbreeding depression are severe (Barrett, 1988; Grosberg & Hart, 2000; Leedale et al., 2020; Pusey & Wolf, 1996; Valbuena-Urena et al., 2017; Wheelwright & Mauck, 1998). Alternatively, traits that actively promote inbreeding via self-fertilization or a preference for related mates are predicted to evolve when mates are scarce, or inbreeding depression is low (Duthie & Reid, 2016; Goodwillie et al., 2005; Jarne & Auld, 2006). Even still, under a broad set of conditions neither active inbreeding avoidance nor preference may be favoured (Kokko & Ots, 2006; Puurtinen, 2011; Waser et al., 1986), suggesting that levels of inbreeding might often reflect genetic subdivision and passive inbreeding tolerance. For many organismal groups, the influence of passive and active processes on the overall magnitude of inbreeding is not well understood, especially for those with less common forms of reproduction. Examining the interactions between demography, the relatedness among conspecifics, and random and non-random mating is key to understanding the ecological and evolutionary factors shaping mating systems.

Extensive theory and empirical work have examined the evolutionary transition from outcrossing to predominant self-fertilization and the connections between demography, self-incompatibility, mate availability, and the wide variation in selfing rates that differentiate mating systems within hermaphroditic species (Auld & de Casas, 2013; Carlon, 1999; Cutter, 2019; Escobar et al., 2011; Goodwillie et al., 2005; Jarne & Auld, 2006; Kalisz & Vogler, 2003; Whitehead et al., 2018). This transition is best characterized in seed plants, where the breakdown of self-incompatibility, reductions in the spatial and/or temporal distance between anthers and pistils (i.e. herkogamy and dichogamy), and closed flowers (i.e. cleistogamy) evolve to increase self-fertilization in isolated locations where mates and pollen are limiting (Barrett et al., 1996; Goodwillie et al., 2005; Pannell, 2015). Mating among relatives (i.e. biparental inbreeding) is predicted to facilitate the transition towards predominant self-fertilization by reducing the severity of inbreeding depression (Porcher & Lande, 2016; Uyenoyama, 1986). Furthermore, theory predicts that biparental inbreeding in itself should be common when there are direct costs to avoiding related mates or if selection against inbred progeny is weak (Duthie & Reid, 2016; Kokko & Ots, 2006; Waser et al., 1986). However, models predicting that biparental inbreeding may be favoured under a broad set of conditions are disconnected from the few taxonomic groups where passive tolerance of or active preference for biparental inbreeding have been explicitly identified in nature (Bilde et al., 2005; Jamieson et al., 2009; Nichols et al., 2014; Pike et al., 2021; Reynolds et al., 2014; Szulkin et al., 2013). Distinguishing the random and non-random components of inbreeding, particularly in species with the potential to actively avoid or prefer related mates, is needed to identify the conditions driving mating system variation across taxa.

A survey of inbreeding coefficients (F_{IS}) suggests that the magnitude of inbreeding in sessile marine invertebrates is on par with terrestrial seed plants, and that variation in inbreeding across species can be explained by differences in reproductive and dispersal traits (Olsen et al., 2020). Because of sessile adult stages, the dispersal, mixing, and recruitment of free-swimming planktonic larvae is a critical determinant of demographic openness and the potential for inbreeding in many marine invertebrates (Addison & Hart, 2005; Hixon et al., 2002; Knowlton & Jackson, 1993; Olsen et al., 2020). In members of this group, limitations to dispersal due to natal philopatry or the collective movement of sibling larvae often place related conspecifics in close proximity and increase the likelihood of inbreeding (Blanquer et al., 2009; Burgess et al., 2016; Calderon et al., 2007; Hellberg, 1994, 1996; Yund & O'Neil, 2000). Yet, allorecognition systems and the ability to transport dispersive gametes in ocean currents suggest that traits affecting the tendency to avoid, tolerate, or prefer related mates contribute meaningfully to the strength of inbreeding in marine invertebrates just as self-incompatibility and floral traits regulate self-fertilization in seed plants (Olsen et al., 2021). However, the extent to which elevated inbreeding in sessile marine invertebrates is due to active non-random mating or passive random mating in subdivided populations is not well characterized.

Similar to wind pollinated plants, spermcasting marine invertebrates rely on oceanic currents as an abiotic external vector to transport male gametes to conspecifics for subsequent uptake and internal fertilization (Bishop & Pemberton, 2006; Johnson & Yund, 2009; Olsen et al., 2021; Pemberton et al., 2004). Despite sessile adult stages, the casting of dispersive sperm can contribute to gene flow in these invertebrates (Grosberg, 1991; Yund, 1995), and in the absence of competition, colonies can fertilize the eggs of conspecifics separated by hundreds of meters (Yund et al., 2007). This is in part because the spermatozoa of these species remain viable for hours or days (Bishop, 1998; Johnson & Yund, 2004) and because internal fertilization is efficient due to the ability to collect, filter, and concentrate sperm from the water column (Pemberton et al., 2003). Moreover, somatic–gametic recognition systems comparable to the pollen–pistil self-incompatibility systems of terrestrial plants have been described in these species that prevent self-sperm and the sperm of relatives from fertilizing retained eggs (Bishop, 1996; Bishop et al., 1996; Scofield et al., 1982). However, like the self-incompatibility systems of seed plants, the presence and effectiveness of these recognition systems are evolutionarily labile and vary among and within species (Grosberg & Hart, 2000; Nydam, 2020).

The potential to actively limit or magnify inbreeding makes spermcasting invertebrates an interesting group to assess the influences of non-random and random mating on the overall

magnitude of inbreeding in these species. We employed a natural experiment with the hermaphroditic colonial ascidian *Lissoclinum verrilli* in qualitatively accessible and isolated locations to test if levels of inbreeding were (1) less than expected given random mating within sites because of active attempts to avoid related mates and limit inbreeding, (2) as predicted by genetic subdivision, random mating within sites, and passive inbreeding tolerance, or (3) greater than expected given random mating within sites due to active attempts to promote inbreeding via self-fertilization or a preference for related mates.

2 | MATERIALS AND METHODS

2.1 | Overview

We evaluated genetic variation among *L. verrilli* colonies and their offspring in sites with differing degrees of spatial isolation to make inferences about philopatric local retention, relatedness, and the magnitude of inbreeding. We employed "mixed mating" progeny arrays, a tool primarily developed to estimate self-fertilization in seed plants (Ritland, 2002), to quantify interpopulation variation in inbreeding and distinguish self-fertilization from biparental inbreeding. Moreover, we simulated progeny arrays with the genotypes of maternal colonies and those of randomly selected sympatric conspecifics without regard to diffusion/advection to evaluate if levels of inbreeding were different from what would be expected given genetic subdivision and random mating within sites.

2.2 | Study species

Lissoclinum verrilli is a colonial ascidian in the family Didemnidae with a documented range that extends throughout the Caribbean and into the Gulf of Mexico (Rocha et al., 2012; Van Name, 1945). This species reproduces sexually on a lunar cycle via spermcasting, in which sperm is released into the sea and then captured for internal fertilization and embryonic development. Colonies maintained in isolation continue to produce biparental offspring for several weeks suggesting that maternal colonies store sperm for some time. Mature tadpole-like larvae develop from a maternal energy source and are released after approximately 12 days. In the laboratory, released larvae settle and metamorphose within minutes. Gene flow as a result of rafting on drifting debris is unlikely in *L. verrilli*, as fragmentation and reattachment to novel substrates in a laboratory setting are often unsuccessful in this species. The zooids of *L. verrilli* (i.e. the physiological units of ascidian colonies) are hermaphroditic, but a somatic–gametic recognition system that blocks self-sperm and may permit mate choice based on the relatedness of conspecifics has been described in a species of the same family (i.e. *Diplosoma listerianum*; Bishop, 1996; Bishop et al., 1996; Pemberton et al., 2004).

2.3 | Sampling

We sampled *L. verrilli* colonies April–October in the years 2016–2019 from 12 sites in the northeastern Gulf of Mexico. Sampling sites (~10–22 m depth) consisted of natural limestone hard-bottom reefs ("Allegedly" and "Sargassum"), artificial concrete substrates ("Two Dogs Reefballs," "Carrabelle Three Mile Culverts," "K Tower," "O Tower"), and a man-made inlet rock jetty ("St. Andrews State Park") (Table S1). We selected these sites because they harboured the study species in varying densities and differed qualitatively in the degree of isolation from other substrates. Natural reefs in this region consist of a patchwork of limestone outcroppings separated by small spits of sand, and sampling sites on these reefs were in close proximity to other outcroppings inhabited by *L. verrilli* (<10 m). In comparison, artificial substrates were surrounded by large expanses of sand without hard substrate close by. Differences in the spatial isolation of natural and artificial sites in this region are apparent, as artificial reefs are purposefully deployed in locations that lack the natural physical structure to supplement fisheries limited by hard substrate (Strelcheck et al., 2005). Artificial substrates were sampled because their remote locations were known and permitted an assessment of inbreeding in geographically isolated locations where the relatedness among conspecifics was likely to be elevated. Isolated artificial sites and accessible natural substrates did not differ significantly in area or in the density or size of *L. verrilli* colonies. At each site, transect tape was extended along the length and width of the continuous substrate and the position and size of each *L. verrilli* colony were recorded. Sampled sites ranged in size from 5 × 3 m to 19 × 7 m. An effort was made to locate every *L. verrilli* within the sampling area and each physically distinct colony was sampled by excising a tissue sample (~5 × 5 cm).

In total, 144 *L. verrilli* colonies were sampled across 12 sites. Tissue samples of *L. verrilli* were transported to Florida State University, fixed in 95% ethanol, and maintained at −20°C. Subsequently, samples were dissected to isolate adult zooids and brooded offspring for DNA extraction. Tissue samples from seven of the 12 sites contained internally developing offspring, likely because sampling occurred in the window after fertilization but prior to the release of offspring. The developmental stage of offspring varied within tissue samples and ranged from newly formed embryos to fully developed larvae nearing release, suggesting that some offspring may have been the product of distinct spawning events. Approximately 15 larvae at the most advanced developmental stage were dissected from three maternal colonies from each of the seven sites.

2.4 | DNA extraction, microsatellite design, and amplification

Lissoclinum verrilli adult zooids were digested in a solution of CTAB and proteinase K in a 65°C water bath, and DNA was extracted and

purified with a magnetic-bead protocol (SprintPrep DNA purification kit, Agencourt Bioscience Corporation Beverly). The DNA of larval offspring was extracted and purified using the protocol of the QIAamp DNA Micro Kit (Qiagen). DNA concentrations were quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and with a Qubit broad-range assay (Thermo Fisher Scientific).

A genomic DNA library enriched for microsatellite loci was developed for *L. verrilli* by the Evolutionary Genetics Core Facility at Cornell University. Ninety potential contigs were screened from this library and 10 loci were selected based on repeat motif, product size, and degree of polymorphism (Table S2). Loci were amplified with the Qiagen Type-it Microsatellite PCR Kit under the following cycling parameters: 95°C for 4 min, 30 cycles of 95°C for 30 s, 57°C for 40 s, 72°C for 45 s, 9 cycles of 95°C for 30 s, 53°C for 40 s, 72°C for 40 s, and a final extension of 72°C for 10 min. Fragment analysis was performed by the DNA Sequencing Facility at Florida State University with GeneScan 500 ROX standard (Thermo Fischer Scientific) and alleles were called in the program Geneious 9.1.8 (Biomatters).

2.5 | Population genetics

Allelic count, linkage disequilibrium ($\bar{r}d$), Nei's genetic diversity, Prevosti's genetic distance, and the number of unique multi-locus genotypes detected by the 10 microsatellite loci were assessed in the R statistical program (version 4.1.2) with the package "poppr" (Kamvar et al., 2021). Spatial patterns of gene flow were evaluated by quantifying pairwise genetic differentiation between sites with Nei's F_{ST} in the package "hierfstat" (Goudet et al., 2020), and the distance between sites, measured with their G.P.S. coordinates and the haversine great-circle distance formula. Because some sites were sampled in different years and temporal variation could confound spatial patterns, genetic differentiation over time in closely situated sites was also assessed. Specifically, pairwise genetic differentiation among four sites within the reef system "Allegedly" was evaluated over three consecutive years to test if genetic differences increased over the sampling period.

Occasionally, identical multi-locus genotypes were detected in physically distinct colonies that were in close proximity (<1 m). These putative clonemates were likely formed by a regressing colony that split into multiple smaller colonies rather than asexual larval propagation (*laboratory-based observation*), and were treated as a single genetic individual.

2.6 | Assignment tests

Assignment tests were used to estimate the strength of philopatric local retention within sites with the program GENECLASS 2 (Piry et al., 2004). This program uses the multi-locus genotypes of individuals to probabilistically assign or exclude reference populations as possible sites of origin. The most probable site of origin was assigned to each *L. verrilli* colony with the likelihood criteria of Rannala and Mountain (1997), and the proportion of individuals reassigned to their sampling location was estimated for each site. This approach is comparable to those with probabilistic cutoffs (Berry et al., 2004) and was used here because we were interested in associations among philopatry, isolation, and inbreeding across all sites, rather than dispersal patterns within any single site. Offspring genotypes were included in assignment tests to assess the capacity of the analysis to correctly identify sites of origin.

2.7 | Relatedness

The degree of relatedness among *L. verrilli* colonies was estimated in the program COANCESTRY (Wang, 2011a). This program implements seven estimates of relatedness that differ in the methodology used to infer identity by descent from allelic states. To select and calibrate the metric used in this study, estimates of relatedness were compared to theoretical expectations in observed mother–offspring pairings where recent coancestry was known. Because pairwise estimates are interdependent (e.g. the relatedness of Mother A to offspring B is not independent of the relatedness between Mother A and offspring C), the means of pairwise estimates were compared. For example, with M maternal colonies let m (i) be maternal colony i and o (i, j) be the *jth* offspring of maternal colony i. Let n (i) be the number of offspring of maternal colony i so that there are n (i) of o (i, j) and M such families. For each m (i), the relatedness with each o (i, j) was estimated and the mean of these pairwise values was calculated (r'). The mean of r' across all maternal colonies (R) was compared among estimators.

Limitations in the resolution of genetic markers and/or inbreeding may bias estimates of relatedness compared with theoretical expectations (Weir et al., 2006). To explore this, the degree of relatedness between maternal colonies and their offspring were evaluated in sites identified as non-inbreeding ($S_S < 0.25$) and inbreeding ($S_S > 0.25$) (see below for how site-level inbreeding was quantified). A resampling with replacement approach was used to populate M families with the same distribution of offspring number as the actual dataset (non-inbreeding sites $n = 9$ maternal families with 52 offspring; inbreeding sites $n = 12$ maternal families with 66 offspring) and r' and R were calculated. Resampling with replacement and averaging was repeated 1000 times to create a bootstrapped distribution of R in non-inbreeding and inbreeding sites. The metric with R closest to the expected value of relatedness for non-inbreeding mother–offspring pairs (i.e. 0.5) and with the smallest 95% confidence interval was used for analyses incorporating relatedness (Table S3). R in non-inbreeding sites was used to conservatively adjust the threshold values of relatedness expected between non-inbred first cousins and half-siblings in this dataset. For each site, 95% confidence intervals of mean relatedness among colonies were estimated by resampling genotypes with replacement in the program.

2.8 | Inbreeding

Levels of inbreeding were measured with progeny arrays in the program MLTR (Ritland, 2002). This program compares allelic frequencies in parental and offspring generations to quantify the total strength of inbreeding due to both self-fertilization and biparental inbreeding and distinguishes their relative contributions to the mating system. We quantified inbreeding in the seven sites where offspring were collected with either the metapopulation as the reference frame or each site as its own reference. Total inbreeding was measured using the single locus estimate of self-fertilization (i.e. 1 – the single locus estimate of outcrossing) with the genotypes of known maternal colonies and all potential sires in each site. In analyses with the metapopulation as the reference frame, each site was identified as a grouping variable and allele frequencies in sperm (pollen) and egg (ovule) pools were estimated separately due to the presence of genetic structure. Biparental inbreeding was distinguished from self-fertilization by genetic exclusion (i.e. the offspring genotype contains an allele that is not present in the maternal genotype) and with metrics developed to identify biparental inbreeding (i.e. t_m–t_s) and estimate the fraction of total inbreeding due to mating among relatives (i.e. 1–r_s; Ritland, 2002).

To evaluate if levels of total inbreeding differed from those expected given genetic subdivisions and random mating within sites, we compared estimates of inbreeding to simulations in each site. For each maternal colony–offspring pair we randomly selected a sire by sampling with replacement from the pool of potential sires within their sampling location. Simulated offspring genotypes were then generated based on the genotypes of the maternal colony and the randomly selected sire. Thus, simulations predicted random biparental mating within sites and did not incorporate other factors such as sire proximity or size. The simulated dataset contained the same distribution of offspring number among maternal families and was evaluated in MLTR with the same parameters as the original analysis. We considered measurements of inbreeding to be different from simulations when their standard deviations did not overlap. For measurements and simulations, standard deviations of mating system parameters were estimated in the program by resampling maternal families with replacement 1000 times.

2.9 | Reference frames for estimating relatedness and inbreeding

Relatedness and inbreeding are relative quantities that can be measured with varying reference frames including those at the population or metapopulation scale (Shields, 1982; Wang, 2011a, 2011b, 2014). We quantified relatedness and inbreeding with the metapopulation as a reference frame and each site as its own reference in separate analyses. Analyses with the metapopulation as the reference frame incorporate the influence of genetic subdivision and relatedness due to shared ancestry in the recent and more distant past. Estimates quantified with site-by-site references exclude shared ancestry and inbreeding due to genetic subdivision. Because our sampling purposefully targeted isolated locations to evaluate the combined effects of genetic subdivision and either random or non-random mating (i.e. inbreeding avoidance, self-fertilization, or preference for related mates), we focus on estimates quantified at the metapopulation scale and refer to the results of site-by-site analyses in the supplement (Figures S1, S2, S3, S4 and S5).

2.10 | Statistical analyses

Analyses in this study focused on evaluating the spatial extent of gene flow, and associations between qualitative isolation, genetic diversity, philopatric local retention, relatedness, census size, and inbreeding. Importantly, genetic estimates of philopatry and relatedness both incorporate allele frequencies and genetic diversity to make inferences about the source of immigrants and the amount of coancestry shared among individuals. Thus, there are methodological as well as biological reasons why these estimates are likely to be correlated. We incorporated these metrics to demonstrate that differences in demographic openness across sites were supported by multiple, albeit correlated estimates.

Genetic differentiation among *L. verrilli* colonies in space and over time was assessed by testing the correlation between pairwise genetic differentiation measured with the linear approximation $F_{ST}/1–F_{ST}$ and either the logarithm of the distance between sites or the number of years between sampling events with a Mantel test in the R package "ade4" (Dray et al., 2020). Nei's genetic diversity, mean genetic distance, the proportion of colonies identified as philopatric, and the mean degree of relatedness among colonies on isolated and accessible sites were compared with census size as a covariate in separate analyses in the R package "car" (Fox et al., 2020). Genetic distances between maternal colonies and their offspring were compared across sites with maternal colonies nested within the site in a nested ANOVA. The association between the magnitude of total inbreeding and the relatedness among *L. verrilli* colonies was evaluated with an errors-in-variables (EIV) regression in the R package "eivtools" (Lockwood, 2018). EIV regression accounts for error in the measurement of an independent variable and was used here because relatedness among individuals varied within sites. Linear regression was used to evaluate the relationship between inbreeding and population census size. The assumptions of inferential tests were confirmed, or data were transformed to meet assumptions prior to reporting results.

3 | RESULTS

From the 144 *L. verrilli* colonies sampled across 12 sites, we detected 137 unique multi-locus genotypes. All repeated genotypes occurred in colonies separated by less than a meter, suggesting that clonemates were formed by colony regression. After consolidating clonemates, linkage disequilibrium in the adjusted dataset was low

($\overline{rd} < 0.01$), indicating that the 10 microsatellite loci segregated independently. Genetic differentiation among colonies was positively correlated with the distance between sites (Mantel test; correlation = 0.669; $p = 0.01$; Figure 1). Moreover, genetic differentiation among colonies sampled in consecutive years within the same reef system was not significantly different from zero (F_{ST} 0.008–0.049), and the magnitude of differentiation was not associated with the number of years between sampling (Mantel test; correlation = 0.310; $p = 0.22$).

Assignment tests correctly identified the site of origin in 94% of offspring (111/118) collected from 7 sites, suggesting that marker resolution was sufficient for estimating rates of philopatric local retention in *L. verrilli* colonies with reasonable accuracy. Estimates of relatedness in mother–offspring pairs were similar across the seven metrics tested (Figure 2a). The metric described by Wang (2002) had the best combination of accuracy and precision relative to the expectation for non-inbreeding mother–offspring pairs (Table S3) and was used in analyses evaluating relatedness among *L. verrilli* colonies. The mean of mother–offspring relatedness estimates in non-inbreeding sites (Figure 2b; $R = 0.547 \pm 0.077$; ±95% CI) was only slightly biased above the expectation (i.e. 0.5), indicating that marker resolution was sufficient to accurately estimate relatedness among *L. verrilli* colonies. The deviation between *R* in non-inbreeding sites and the expectation in mother–offspring pairs (i.e. 0.047) was used to conservatively adjust the expected relatedness between first cousins and half-siblings in this dataset (i.e. adjusted thresholds Figures 3 and 4).

The genetic diversity of *L. verrilli* colonies varied significantly among qualitatively accessible and isolated sites (Figure 3). Nei's genetic diversity and mean genetic distance among colonies were significantly greater on accessible substrates compared with those that were isolated ($H_{expected}$; ANCOVA; $df = 1$, $F = 7.445$; $p = 0.023$; genetic distance; ANCOVA; $df = 1$; $F = 9.098$; $p = 0.015$) and were not associated with census size ($H_{expected}$; $df = 1$, $F = 1.136$, $p = 0.314$; genetic distance; $df = 1$, $F = 0.597$, $p = 0.460$). Concordantly, estimates of philopatry and relatedness among *L. verrilli* colonies also varied considerably across sites (Figure 3c,d). Estimates of the proportion of philopatric colonies were greater on isolated substrates compared with accessible sites (ANCOVA; $df = 1$; $F = 13.745$; $p = 0.005$) and were not associated with census size ($df = 1$; $F = 0.104$; $p = 0.755$). Similarly, estimates of mean relatedness among colonies were higher on isolated substrates compared with accessible sites (ANCOVA; $df = 1$; $F = 7.456$; $p = 0.023$) and were also not associated with census size ($df = 1$; $F = 0.984$; $p = 0.347$).

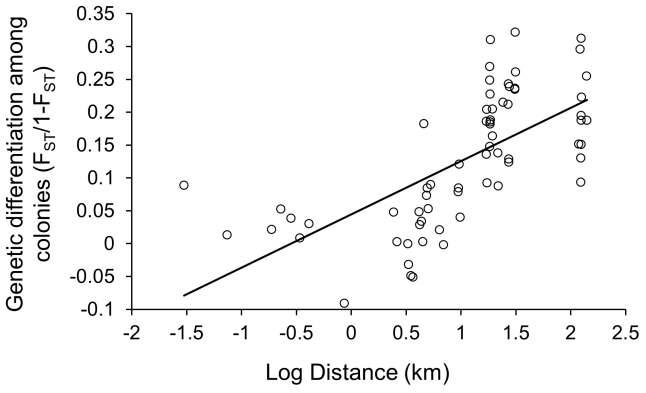

FIGURE 1 Pairwise genetic differentiation among sites harbouring *Lissoclinum verrilli* colonies was positively associated with the distance between sampling locations ($n = 12$, 66 comparisons).

In total, 118 offspring dissected from 21 maternal colonies were evaluated in progeny arrays to quantify levels of inbreeding across 7 sites (Table 1). Genetic distances between maternal colonies and their offspring varied significantly among sites (Nested ANOVA on ranks; $df = 6$, $F = 5.395$, $p < 0.001$; Figure S3a), suggesting that levels of inbreeding differed in the locations sampled. Eleven offspring harboured multi-locus genotypes that were identical to the maternal colony (i.e. genetic distance = 0). These offspring were possibly the product of asexual reproduction, self-fertilization, or close biparental inbreeding. An additional 5 offspring were genetically distinct from the maternal colony (i.e. genetic distance >0) but their multi-locus genotypes could have been explained by either self-fertilization or biparental inbreeding. The majority of offspring (102/118) harboured non-maternal alleles in their multi-locus genotypes indicating they were the product of biparental reproduction rather than asexual propagation or self-fertilization.

Estimates of total inbreeding quantified with all 118 offspring and a metapopulation reference frame were highly variable and ranged from 0.015 to 0.755 (Figure 4a). These estimates were positively associated with the mean relatedness among colonies (EIV regression; slope = 0.895; $p = 0.029$; Figure 4a) but were not associated with population census size (linear regression; F (1, 5) = 2.288; slope = 0.014; $p = 0.191$; Figure 4c). Furthermore, the standard deviations of these estimates overlapped with simulations of random mating in 6 of 7 sampled locations. Concordantly, estimates of total inbreeding that excluded the effects of genetic subdivision in site-by-site analyses were not significantly different from zero in 5 of 7 sites (Figure S5a). Thus, both analyses suggest that levels of inbreeding were not different from those expected given genetic subdivision and random mating in most sites.

Biparental reproduction confirmed with non-maternal alleles and supported by MLTR estimates (i.e. $t_m - t_s$ and $1 - r_s$) was more common than potential self-fertilization in all sites evaluated (Table 1). After removing the 16 offspring that could have been produced by self-fertilization, mother–offspring genetic distances still varied significantly among sites (Nested ANOVA on ranks; $df = 6$, $F = 3.227$, $p = 0.007$; Figure S3b). Furthermore, estimates of inbreeding quantified with the metapopulation as reference were still significantly associated with the mean relatedness among *L. verrilli* colonies (EIV regression; slope = 0.806; $p = 0.024$; Figure 4b), but not census size (linear regression; F (1, 5) = 0.319; slope = 0.005; $p = 0.597$). Finally, the standard deviations of total inbreeding estimates in biparental offspring overlapped with simulations of random mating in all 7 sites

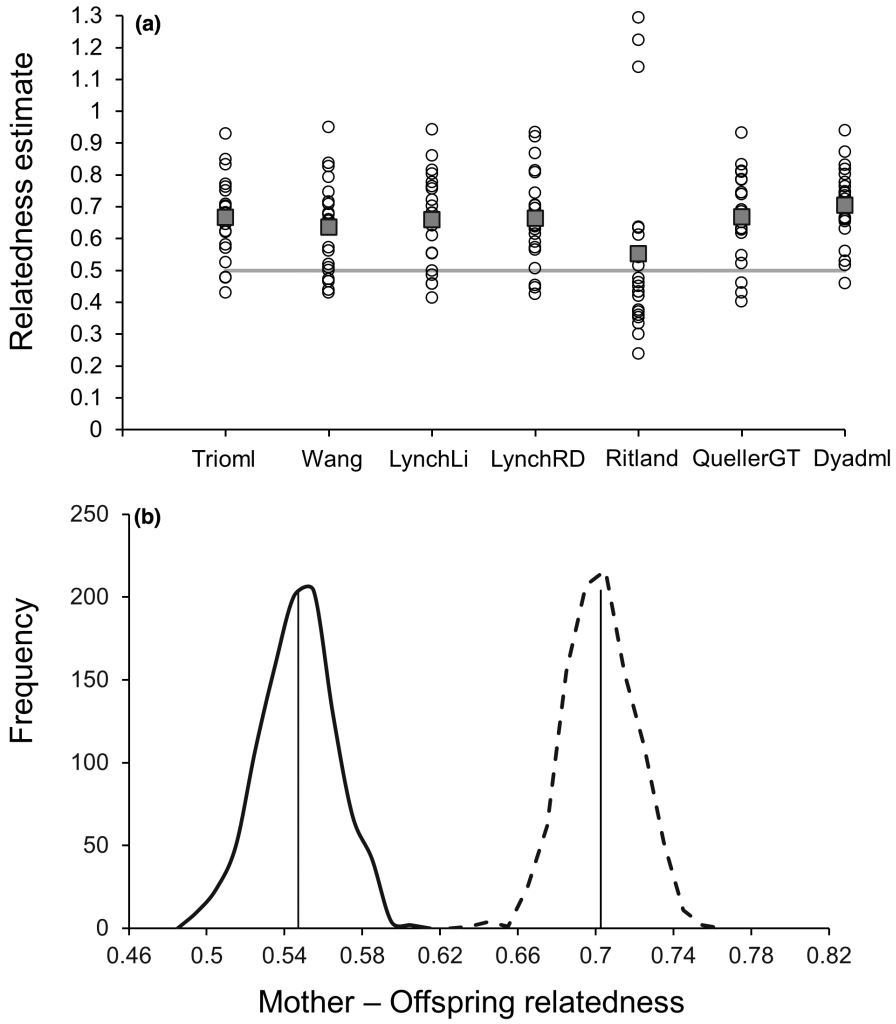

FIGURE 2 Estimates of pairwise relatedness between *Lissoclinum verrilli* maternal colonies and their offspring. In (a), open circles represent independent pairwise estimates of relatedness (r') between maternal colonies and their offspring ($n = 21$ maternal families) with the seven metrics available in the program COANCESTRY. Grey squares represent the mean of independent estimates (R). The grey horizontal line represents the expected value of relatedness between non-inbreeding mothers and offspring. In (b), distributions of bootstrapped relatedness between maternal colonies and their offspring in sites identified as non-inbreeding ($S_s < 0.25$; solid line) and inbreeding ($S_s > 0.25$; dotted line) were measured with the Wang (2002) estimate. Vertical lines are the means of bootstrapped estimates.

(Figure 4b) and were not significantly different from zero in site-by-site analyses (Figure S5b).

4 | DISCUSSION

We found dramatic differences in the demography, relatedness among colonies, and magnitude of inbreeding across populations of *L. verrilli*. Sampling locations close to exogenous sources of conspecific larvae and sperm had comparatively open demographics and harboured colonies that on average shared little or no relatedness (Figure 3). In more isolated locations, genetic signatures of philopatric local retention and mean relatedness on the order of first cousins and half-siblings were evident. Levels of total inbreeding estimated with progeny arrays tracked with the mean relatedness of conspecifics across sampling locations irrespective of census size (Figure 4). Predominately, estimates of total inbreeding were not different from the predicted influence of genetic subdivision and random mating within sites, suggesting that *L. verrilli* colonies primarily tolerated related mates rather than actively avoided or preferred inbreeding. Our results emphasize the effects of demographic isolation on the genetic composition of species with limited dispersal and coincide with models predicting that tolerance of biparental inbreeding may be common across a variety of reproductive modes and ecological conditions.

4.1 | Gene flow, demographic openness, and relatedness

Genetic differentiation among *L. verrilli* colonies was strong over comparatively small spatial scales, and sites separated by 18 km or more were consistently distinct (Figure 1). Populations of spermcasting marine invertebrates with indirect development can be homogenized by gene flow via free-swimming larvae and/or the planktonic transfer of sperm (Grosberg, 1991; Yund, 1995). Yet, the spatial extent of migration is generally restricted in members of this group because the cumulative time spent in the plankton is shorter than for other forms of development where larvae must feed for weeks prior to settlement (Selkoe & Toonen, 2011). While species with reduced larval duration tend to exhibit increased genetic subdivision, many exceptions point to the importance of larval behaviour, habitat specificity, adult rafting, and water movement in the spatial extent of gene flow in marine invertebrates (Ayre et al., 2009;

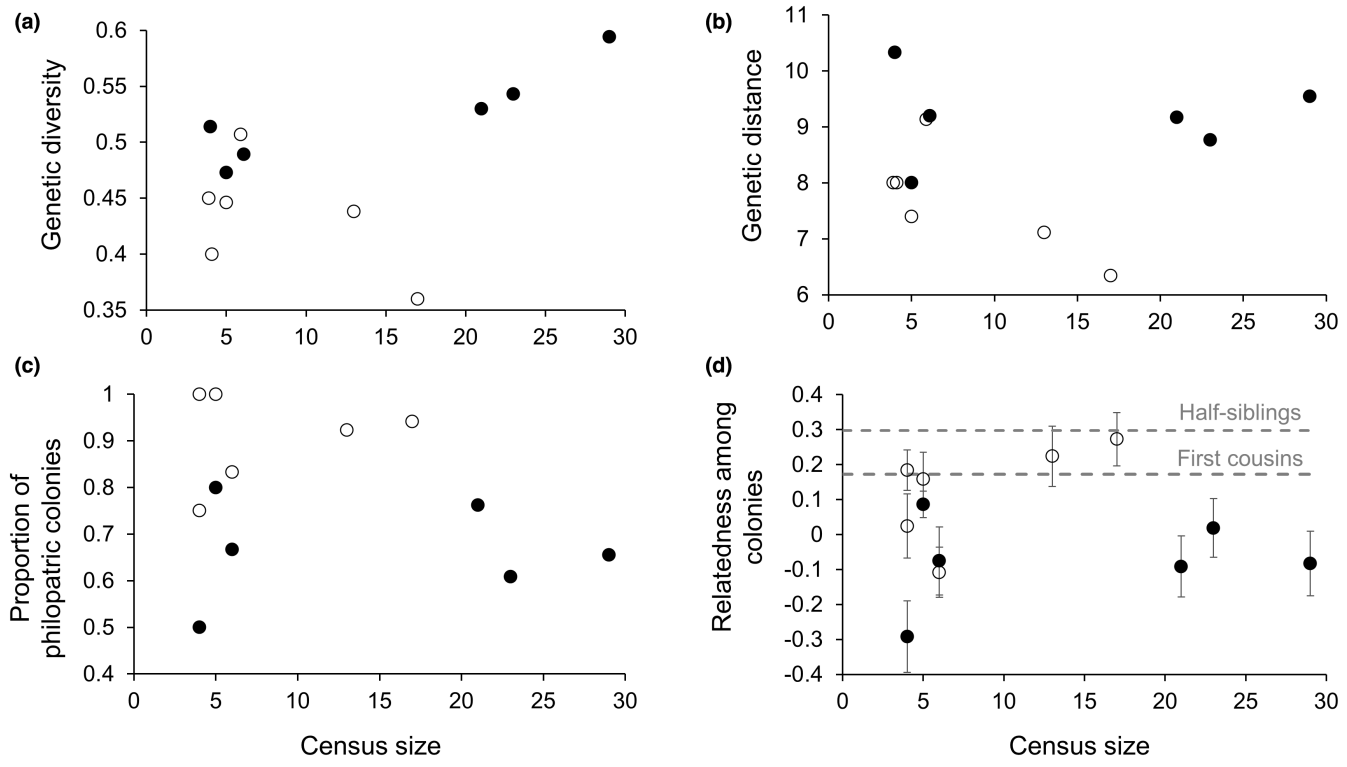

FIGURE 3 Estimates of genetic diversity, philopatric local retention, and relatedness among *Lissoclinum verrilli* colonies in qualitatively accessible natural ($n = 6$; closed symbols) and isolated artificial ($n = 6$; open symbols) sites. Colonies in isolated sites had significantly lower Nei's genetic diversity (a) and mean genetic distances (b), and significantly greater estimates of local retention (c) and mean relatedness (d) compared with colonies in accessible sites. Error bars represent 95% confidence intervals of mean relatedness. Dashed lines represent the predicted relatedness of non-inbred first cousins and half-siblings following calibration with observed estimates of mother–offspring relatedness.

Dupont et al., 2009; Olsen et al., 2016; Severance & Karl, 2006; Shanks, 2009).

The distance over which populations of *L. verrilli* began to differentiate was surprisingly small even relative to other species with the same reproductive and developmental modes (Dupont et al., 2009; Duran et al., 2004; Ledoux et al., 2010). Temporal differences in sampling are an unlikely explanation for the genetic differentiation detected among sites, as *L. verrilli* colonies often persist for several years and F_{ST} between closely situated sites sampled over multiple years was low. Instead, isolation over short distances suggests there are broad restrictions to dispersal in this species, either due to traits that limit larval longevity or because the patchiness of hard substrate and physical oceanography in the sampling region restrict the movement of larval and gametic propagules.

Given the limitations to migration detected in *L. verrilli*, it might be expected that populations of this species would be ubiquitously closed even at small spatial scales. However, demographic openness at the scale of meters varied markedly in sites that differed qualitatively in spatial isolation (Figure 3). Sampling areas with other substrates nearby were comparatively open with 20%–50% of colonies likely originating from outside the collection site. In comparison, sampling areas located greater distances from exogenous sources of sperm and larvae harboured colonies with reduced genetic diversity and were completely or nearly completely closed with all or most colonies reassigned to the ~8×8 m substrate from which they were collected (Figure 3c). Analyses evaluating the degree of relatedness among *L. verrilli* colonies also support stark differences in patterns of demographic openness among sites (Figure 3d). Estimates of relatedness ranged from values indicating that colonies shared little or no recent common ancestry to those suggesting that individuals were, on average, first cousins or half-siblings even after conservative calibration. While estimates of genetic diversity, philopatric local retention, and mean relatedness were significantly different across spatially isolated artificial substrates and accessible natural sites, these measures also overlapped, pointing to a spectrum of demographic openness among sampling locations. Variations within our isolated and accessible classifications may represent differences in the number of generations since colonization or spatial differences in the transport of gametes and larval offspring. Isolated sites with elevated genetic diversity may have been colonized more recently or situated in areas where larval transport was common due to water currents. Alternatively, sites we considered accessible may have been effectively closed to incoming migrants because of the local oceanography. Closed dynamics and shared ancestry within populations have been reported in reef fish and invertebrates with planktonic larvae that develop for weeks (Burgess et al., 2014; Hogan et al., 2012), signifying that philopatric local retention over small spatial scales in *L. verrilli* can partially be attributed to a short

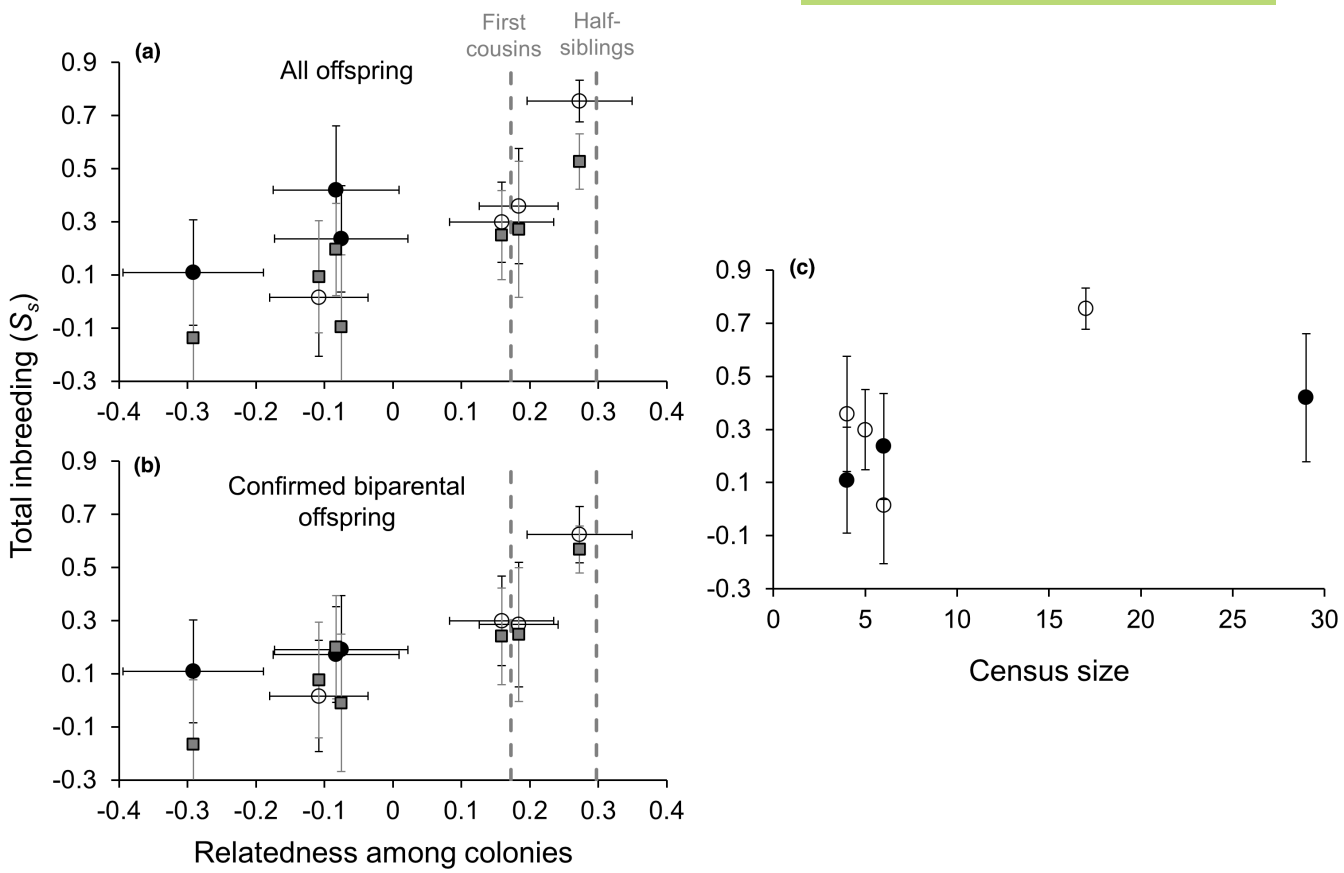

FIGURE 4 Total inbreeding (S_s) in *Lissoclinum verrilli* was positively associated with the mean relatedness among conspecifics (a,b), but not census size (c) across seven sites. Estimates of total inbreeding incorporating all offspring ($n = 118$) were not significantly different from simulations of random mating within sites, represented by grey squares and error bars, in 6 of 7 sites (a). Estimates of total inbreeding incorporating only offspring where biparental reproduction was confirmed ($n = 102$) were not significantly different from simulations of random mating in all seven sites (b). Total inbreeding and relatedness are quantified with the metapopulation as the reference frame. Closed symbols are accessible natural sites, open symbols are isolated artificial sites. Error bars along the *y*-axis represent standard deviations of total inbreeding and error bars along the *x*-axis represent 95% confidence intervals of mean relatedness. Dashed lines represent the predicted relatedness of non-inbred first cousins and half-siblings following calibration with observed estimates of mother–offspring relatedness.

TABLE 1 Mating system estimates across sites quantified with the metapopulation as reference

Site	Sample size	Total inbreeding (S_s)	Selfing excluded	$t_m - t_s$	$1 - r_s$
Allegedly-4	3 (15)	0.420 ± 0.241	9/15 (0.6)	0.034 ± 0.114	0.703 ± 0.207
Carrabelle Three Mile Culverts	3 (15)	0.015 ± 0.221	15/15 (1.0)	0.215 ± 0.100	1.200 ± 0.368
K Tower-West Wall	2 (15)	0.359 ± 0.217	13/15 (0.9)	0.208 ± 0.201	0.903 ± 0.069
O Tower	4 (21)	0.755 ± 0.078	14/21 (0.7)	0.374 ± 0.124	1.067 ± 0.100
Sargassum-1	3 (16)	0.236 ± 0.200	15/16 (0.9)	0.178 ± 0.133	1.042 ± 0.268
Sargassum-2	3 (21)	0.109 ± 0.199	21/21 (1.0)	0.309 ± 0.084	1.194 ± 0.160
Two Dog Reefballs	3 (15)	0.299 ± 0.151	15/15 (1.0)	0.499 ± 0.361	1.187 ± 0.054

Note: The sample size of maternal colonies with the number of offspring in parentheses, total inbreeding measured with the single locus estimate of selfing (S_s), the number of offspring where self-fertilization was excluded by the presence of non-maternal alleles with the proportion in parentheses, and total inbreeding due to mating among relatives ($t_m - t_s$ and $1 - r_s$).

larval duration lasting only minutes or hours. Nonetheless, we found considerable variation in the demographic openness of *L. verrilli* populations and leveraged differences in the distribution of relatedness among conspecifics to assess patterns of inbreeding avoidance, tolerance, and preference.

4.2 | Differentiated levels of inbreeding and tolerance of related mates

Comparison with simulations and a positive association between levels of total inbreeding and the mean relatedness among conspecifics

indicate that *L. verrilli* passively tolerated rather than actively avoided or preferred inbreeding in subdivided populations (Figure 4). This pattern is unlike those reported for many species where behavioural and gametic mechanisms are employed to avoid related mates and inbreeding depression (Bretman et al., 2004; Leedale et al., 2020; Pusey & Wolf, 1996; Valbuena-Urena et al., 2017; Wheelwright & Mauck, 1998). Yet, avoiding inbreeding can also reduce fitness, particularly if mating opportunities are missed completely (Duthie & Reid, 2016; Kokko & Ots, 2006), and our results support the theory predicting that tolerance of biparental inbreeding may be more widespread than often expected (Waser et al., 1986).

The genetic composition of *L. verrilli* maternal colonies and their offspring differed significantly across sampling sites, pointing to interpopulation variation in the mating system of this spermcasting invertebrate (Figure 2b and Figure S3a). Outcrossing was the predominant form of reproduction in all sites examined (Table 1), but in a small subset of offspring (14%) asexual reproduction or self-fertilization could not be distinguished from mating among close relatives. Given that nearly all sampled colonies harboured unique multilocus genotypes and clonemates were only found in a spatial configuration consistent with colony regression, we conclude that asexual production of larval offspring was unlikely. Infrequent self-fertilization may have contributed to differences among mating systems of *L. verrilli*, but levels of total inbreeding largely did not differ from the predicted influence of genetic subdivision and random biparental mating (Figure 4a). Furthermore, analyses with potentially selfed offspring removed still detected significant variation in the magnitude of inbreeding across sites (Figure 4b and Figure S3b). Overall, the results suggest that interpopulation variation in mating systems were primarily driven by passive tolerance of related mates in subdivided populations.

Variation in self-fertilization and the repercussions for fitness have commonly been investigated between and within species of hermaphroditic marine invertebrates (Boissin et al., 2008; Carlon, 1999; Carlon & Lippe, 2011; Cohen, 1990, 1996; Fogarty et al., 2012; Heyward & Babcock, 1986; Hughes et al., 2009; Johnson, 2010; Levitan et al., 2004; Phillippi & Yund, 2017; Sherman, 2008). In comparison, there have been far fewer evaluations of biparental inbreeding in this group (but see Palmer & Edmands, 2000; Hoare & Hughes, 2001; Burgess et al., 2019). The current work is one of only a few attempts to distinguish in situ biparental inbreeding avoidance, tolerance, and preference across multiple populations of a marine invertebrate. In a single location, Grosberg (1987) found that spatial limitations in the dispersal of larval offspring and in the effectiveness of spermcasting promote consanguineous mating in the colonial ascidian *Botryllus schlosseri*. Similarly, proximity-dependent mating and genetic subdivision have been identified as important contributors to levels of inbreeding in barnacles and red corals (Ledoux et al., 2020; Plough et al., 2014). Our study differs from previous work in that it addresses the combined influences of genetic subdivision and random or non-random mating on levels of inbreeding across populations of a sessile marine invertebrate.

We found that differences in levels of inbreeding among *L. verrilli* populations were consistent with genetic subdivision coupled with passive tolerance of related mates (Figures 2b and 4). However, our analyses focused on mating system variation at a coarse population level and more nuanced effects associated with mate proximity, colony size, and/or water flow may have gone undetected with our approach based on mean relatedness and levels of inbreeding. For example, related colonies clustered in close proximity could have increased the strength of inbreeding beyond what would be predicted given random mating at the population level. This may partially explain why inbreeding estimates tended to be higher but not significantly different from simulations of random mating in our study. This being said, active attempts to avoid related mates, self-fertilize, or prefer related mates did not shift levels of inbreeding beyond what would be expected given random mating, suggesting that non-random mating was either not prevalent or was inconsequential at this scale.

Differentiated mating systems were evident regardless of population size, as the two sites with the greatest census sizes had the highest levels of inbreeding (Figure 4c). This pattern is opposite to what would be expected if biparental inbreeding were constrained to small ephemeral populations in this species. Rather, our results support the theory predicting that tolerance of biparental inbreeding may be common within and across species with diverse modes of reproduction, especially those with limited ability to disperse from relatives and where the costs of avoiding inbreeding may outweigh those of inbreeding depression (Kokko & Ots, 2006; Pike et al., 2021; Waser et al., 1986).

The severity of inbreeding depression and the ancestral state associated with inbreeding avoidance, tolerance, or preference are central to whether tolerance of biparental inbreeding represents an evolutionary adaptation, is a byproduct of selection acting on correlated traits, or is maladaptive in *L. verrilli*. Allorecognition systems are the basis of self-incompatibility and have been implicated in the ability to regulate biparental inbreeding in tunicates and other invertebrates (Bishop, 1996; Bishop et al., 1996; Burgess et al., 2019; Grosberg & Hart, 2000; Nydam, 2020; Pemberton et al., 2004). Under certain conditions, allorecognition systems regulating inbreeding avoidance may breakdown to permit tolerance of biparental inbreeding in a similar fashion to the evolutionary loss of self-incompatibility and the transition from obligate outcrossing to predominant self-fertilization that occurs in terrestrial seed plants under demographic isolation (Olsen et al., 2021; Pannell, 2015). We evaluated biparental inbreeding in populations located at the northern edge of this species' range (Rocha et al., 2012; Van Name, 1945), where inbreeding depression and the costs of tolerating related mates may be reduced (Pujol et al., 2009). Experiments evaluating inbreeding avoidance, tolerance, and preference in the core of *L. verrilli*'s range could test if tolerance of related mates is an adaptation to reduced mate availability at the range edge. Alternatively, tolerance of related mates may have been shaped indirectly by selection acting on other traits associated with fertilization and sperm competition

(Hughes et al., 2009; Levitan & Petersen, 1995). Depending on the severity of inbreeding depression, tolerance of related mates may be maladaptive in *L. verrilli* and biparental inbreeding could have considerable costs similar to those of self-fertilization in long-lived gymnosperms (Husband & Schemske, 1996; Lande et al., 1994; Winn et al., 2011).

Further research evaluating the fitness consequences of inbreeding tolerance is needed to better understand the potential overlap in the evolutionary dynamics of biparental inbreeding and self-fertilization. Biparental inbreeding is becoming better recognized in a subset of animals (Bilde et al., 2005; Jamieson et al., 2009; Nichols et al., 2014; Pike et al., 2021; Szulkin et al., 2013) and is often identified as a facilitator of self-fertilization (Porcher & Lande, 2016; Uyenoyama, 1986), but the evolution of traits that may contribute to the tolerance of biparental inbreeding has not been as well studied as those that enable self-fertilization. Unlike self-fertilization which is known to be adaptive under certain conditions and is associated with the correlated evolution of inbreeding depression, reproductive and dispersal traits, and lifespan (Auld & de Casas, 2013; Cutter, 2019; Lesaffre & Billiard, 2020; Schemske & Lande, 1985), our understanding of the adaptive value of biparental inbreeding is far less complete. Given that mating among related conspecifics is common in marine invertebrates, and that variation in inbreeding is associated with an incredible diversity of reproductive and developmental traits (Olsen et al., 2020, 2021), marine invertebrates offer a substantial opportunity to address the adaptive value of biparental inbreeding and expand the general study of mating systems.

AUTHOR CONTRIBUTIONS

Kevin Olsen: Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (supporting); investigation (lead); methodology (equal); project administration (equal); resources (supporting); software (equal); supervision (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Don Levitan:** Conceptualization (supporting); data curation (supporting); formal analysis (supporting); funding acquisition (lead); investigation (supporting); methodology (equal); project administration (equal); resources (lead); software (equal); supervision (equal); validation (equal); visualization (supporting); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data supporting this manuscript has been submitted to the dryad repository: 10.5061/dryad.51c59zwc4.

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SUPPORTING INFORMATION

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

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