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SUMMARY

1. The freshwater bryozoan *Cristatella mucedo*, in common with other sessile, benthic freshwater taxa, has an unusual life history: sex occurs during a relatively brief period near the start of the growing season, and overwintering occurs in the form of asexually produced dormant propagules (statoblasts). Consistent observed heterozygosity (Ho) deficits in *C. mucedo* populations have previously suggested that inbreeding is common, although a possible contribution of a Wahlund effect to low Ho could not be discounted.
2. We have used microsatellite data in the first study based on codominant markers to genetically characterise maternal colonies and larval offspring of *C. mucedo*. The 'population' represented by the larvae was in Hardy–Weinberg equilibrium, which has previously been found in only one of 39 populations of *C. mucedo*. At least 64% of larvae were the products of outcrossing. We suggest that the unusual early timing of sex may be a strategy to maximise rates of outcrossing within populations of sessile freshwater invertebrates.

Keywords: freshwater bryozoan, life history, observed heterozygosity, outcrossing, Wahlund effect

Introduction

Many cyclically parthenogenetic freshwater invertebrates, including numerous zooplankton, reproduce clonally throughout the summer, thereby obtaining the benefits of prolific asexual reproduction while avoiding the risks associated with mating. At the end of the summer, a bout of sexual reproduction produces genetically variable individuals in the form of resistant propagules that will overwinter and re-establish populations the following year (Bell, 1982; Lynch & Spitze, 1994). Such a life history maximises genetic diversity at the start of each growing season and should therefore increase the likelihood that at least some offspring will be adapted to an environment that may change from one year to the next.

In contrast to the life cycle described above, sessile benthic freshwater invertebrates that reproduce both sexually and asexually have an unusual life history. Taxa within this relatively small group, including freshwater bryozoans, sponges, and the hydrozoan *Cordylophora* spp., undergo a brief period of sexual reproduction relatively early in the growing season and overwinter as asexually produced resistant propagules. The period between the sexual and overwintering phases is marked by extensive clonal reproduction. Of the freshwater species that follow this life history pattern, probably the most extensively studied, at least with respect to population genetics, is the bryozoan *Cristatella mucedo* (Phylum Bryozoa; Class Phylactolaemata).

Cristatella mucedo inhabits discrete lakes and ponds throughout the Holarctic. As with all freshwater bryozoans, *C. mucedo* is hermaphroditic and colonial. A brief period of sex occurs in the early summer, at which time sperm are released into the water column. Following internal fertilisation, larvae are brooded within the colony. Upon release, larvae are motile for a short period before settling. Most reproduction occurs through clonal processes including budding, colony fission, and the production of resistant, buoyant, dormant propagules called statoblasts, which are the only means of overwinter survival. Recent evidence suggests that some initially buoyant statoblasts

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become incorporated in sediments where they may remain viable for periods greater than 1 year, thereby creating a statoblast bank (analogous to a seed bank; Leck, Parker & Simpson, 1989) from which genotypes may be reintroduced into the population (Freeland, Rimmer & Okamura, 2001).

Ecological observations indicate that sexual reproduction in *C. mucedo* may occur irregularly within sites, and is apparently foregone in some years (Okamura, 1997a). Population genetic data, based on microsatellite loci, previously revealed consistently low levels of observed heterozygosity (Ho) in 39 *C. mucedo* populations in Europe and North America (Freeland, Noble & Okamura, 2000a,b; Freeland *et al*., 2001). These Ho deficits were interpreted as evidence for extensive inbreeding, although a Wahlund effect was not discounted as there was a possibility that subdivided populations were being pooled. This may lead to fewer heterozygotes if the subpopulations show variations in allele frequencies. Random amplified polymorphic DNA (RAPD) fingerprints of larvae and parental colonies revealed some evidence of outcrossing in *C. mucedo*, although overall similarity between parent and offspring genotypes remained high (Jones, Okamura & Noble, 1994). The dominant nature of RAPD profiles meant that no information about Ho levels was obtained, and therefore inferences about inbreeding were limited. Here we use the first data based on codominant markers to assess the extent of outcrossing in freshwater bryozoans. Our results have further implications for population processes such as subdivision and mixing of genetically dissimilar cohorts.

Methods

On 10 July 2000 we collected *C. mucedo* colonies from Tufty's Corner, Berkshire, U.K. As in previous studies, we collected only a single colony from clumped distributions in order to decrease the likelihood of sampling clonal replicates that result from colony fission. Each colony was placed in a separate Petri dish. Mature larvae of *C. mucedo* are semitransparent, spherical and approximately 1 mm in diameter. Twenty-five larvae released overnight by 16 colonies were collected the following morning with a pipette and stored at −20 °C in approximately 50 μL of water. Forty adult colonies (16 of which had released larvae) were stored individually in 99% ethanol at 4 °C.

The DNA was extracted as in earlier studies (Freeland *et al*., 2000a), with the exception that larvae were extracted in 20 μL chelex (colony tissue was extracted in 250 μL chelex). Polymerase chain reactions (PCR) for the colonies were as before (Freeland *et al*., 2000a). The PCR for the larvae were scaled down to a 20-μL reaction that included 3 μL of supernatant from the chelex extractions. Unlike previous studies that characterised five loci, only three loci (1.1, 2.2 and 9.4; Freeland *et al*., 1999) were genotyped because of the limited amount of DNA that each larva yielded. The genetic profiles of the larvae were compared with the colonies from which they were released (maternal colony). Outcrossing was inferred when larvae contained an allele(s) that was not found in the maternal colony.

Data from the 25 larvae and 40 colonies were treated as two separate populations and analysed in Popgen (Yeh & Boyle, 1997) to obtain measures of expected and observed heterozygosity (He and Ho), inbreeding (Fis), and presence or absence of Hardy–Weinberg equilibrium using the algorithm by Levene (1949) using a likelihood ratio test. Clonal diversity (D*) for each data set was calculated as the proportion of colonies or larvae that was represented by unique genotypes.

Results

In keeping with the results of previous studies, the microsatellite data revealed heterozygote deficits in the population represented by colonies at Tufty's Corner. The genetic profile based on the 40 colonies showed a mean Ho across the three loci of 0.34 (±0.13), a mean He of 0.64 (±0.13), and Fis values of 0.38 at locus 1.1, 0.64 at locus 2.2, and 0.35 at locus 9.4. These values fall within the ranges found in populations characterised in previous years (Freeland *et al*., 2000a,b, 2001). The likelihood ratio test revealed a lack of Hardy–Weinberg equilibrium at all three loci ($P < 0.001$ in all cases). The total clonal diversity (D*) was 72.5%, which falls within the range of clonal diversity values found in other populations (Freeland *et al*., 2000a,b, 2001).

Analysis of the larval data revealed a mean Ho across the three loci of 0.45 (±0.05), a mean He of 0.57 (±0.11), and Fis values of 0.17 at locus 1.1, 0.27 at locus 2.2 and 0.10 at locus 9.4. D* for all larvae was 72%, and when identical sibs were removed from the data

set, D* = 92.9%. The combined genotypic frequencies of the larvae were not significantly different from the genotypic frequencies expected under random mating according to the likelihood ratio test ($P > 0.05$) for all three loci.

Sixteen of the 25 genotyped larvae (64%) showed strong evidence for outcrossing as they had at least one allele that was not present in the maternal colony (Table 1). The most likely explanation is that these alleles were contributed by sperm with genotypes different to those of the maternal colonies. While sample sizes were insufficient to obtain rigorous estimates of inbreeding, it is likely that our results provide an underestimate of outcrossing. The three

Table 1 Comparison of larval and maternal genotypes

Family	No. of larvae	Material†	Locus 1.1	Locus 2.2	Locus 9.4
1	1	M	215,215	242,242	172,172
		La*	215,**228**	242,242	172,172
2	1	M	216,228	242,242	172,172
		La	216,228	242,242	172,172
3	1	M	206,216	244,244	176,176
		La*	216,**228**	**242**,244	**172**,176
4	1	M	216,216	242,242	172,172
		La*	216,**228**	242,242	172,**175**
5	3	M	216,230	242,242	172,176
		La*	**228**,230	242,**244**	176,176
		Lb	216,216	242,242	172,172
		Lc*	216,216	242,**244**	172,**174**
6	1	M	218,230	242,242	172,176
		La*	**215**,230	242,**250**	176,176
7	2	M	216,230	242,242	172,172
		La	216,216	242,242	172,172
		Lb	216,216	242,242	172,172
8	1	M	216,230	242,242	175,176
		La*	216,230	242,**244**	**172**,175
9	1	M	206,216	250,250	175,175
		La*	216,216	**244**,250	175,175
10	1	M	216,230	244,250	172,176
		La*	216,**228**	244,250	172,**175**
11	3	M	228,228	244,250	172,172
		La*	**216**,228	244,250	172,172
		Lb	228,228	250,250	172,172
		Lc*	228,228	244,**246**	172,172
12	1	M	218,218	244,250	172,172
		La*	218,**228**	250,250	172,172
13	2	M	216,228	244,250	172,172
		La	216,216	244,244	172,172
		Lb	228,228	244,244	172,172
14	2	M	216,216	244,244	175,175
		La*	216,**228**	244,**250**	**172**,175
		Lb*	216,**228**	244,244	**172**,175
15	3	M	216,216	244,244	175,175
		La*	216,216	244,244	**172**,175
		Lb	216,216	244,244	175,175
		Lc	216,216	244,244	175,175
16	1	M	216,216	244,244	172,172
		La*	216,216	244,244	172,**175**

†Material refers to either the maternal colonies (M) or the larvae (L). Larvae with asterisks (L*) are those with evidence for outcrossing. Numbers written in the loci columns refer to the sizes of the amplified alleles. Bold numbers represent alleles in the larvae that were not present in the maternal colonies. The allele size ranges for each locus are comparable with those found in other *Cristatella mucedo* populations in the U.K. (Freeland *et al.*, 2001).

microsatellite loci that we genotyped revealed, in the population of 40 colonies, a total of six alleles at locus 1.1 (with frequencies ranging from 0.025 to 0.33), four alleles at locus 2.2 (with frequencies ranging from 0.013 to 0.46), and four alleles at locus 9.4 (with frequencies ranging from 0.050 to 0.66). Genotyping at a greater number of polymorphic microsatellite loci would probably have detected a greater number of novel bands. No alleles were found in the larvae that were not present in the population of adult colonies. Six of the families included more than one larva (Table 1) and, as there were no more than two novel alleles at each locus within each family, there was no evidence for fertilisation of a single maternal colony by more than one sperm genotype.

Discussion

Contrary to expectations based on previous population genetic studies, a high proportion of larvae were evidently the products of outcrossing. Although we cannot discount the possibility that some larvae resulted from inbreeding or selfing, it is noteworthy that the larvae represent a *C. mucedo* 'population' in Hardy–Weinberg. In previous studies, 39 populations characterised by microsatellites revealed only a single population in Hardy–Weinberg equilibrium, a genetically depauperate population from Hidden Lake, Washington, that revealed only two genotypes (Freeland *et al*., 2000b).

Given the consistent Ho deficits found in previously genotyped populations, it is likely that factors other than, or in addition to, inbreeding are inflating overall levels of homozygosity in *C. mucedo* populations. A possible role of the Wahlund effect was previously acknowledged (Freeland *et al*., 2000a), but initially there was no obvious mechanism for mixing large numbers of individuals from different populations. Inferences of gene flow among sites were low (Freeland *et al*., 2000a,b; Freeland, Romualdi & Okamura, 2000c; Freeland *et al*., 2001), and dispersal overland therefore seemed an unlikely cause of such mixing. Although population structuring within localities can also result in a pronounced Wahlund effect (Bilton, 1992), *C. mucedo* produces buoyant statoblasts which will be distributed around a site by wind, water currents and other vectors, thereby leaving little opportunity for spatial subdivision within a site as suggested by preliminary genetic analysis of a population at Tufty's Corner (J.R. Freeland, unpublished data).

Recent evidence for the reintroduction of genotypes into a *C. mucedo* population from a statoblast bank (Freeland *et al*., 2001) identifies a feasible mechanism for the creation of a Wahlund effect, assuming that the statoblast banks contain an admixture of propagules that originated in temporally isolated populations with different allele frequencies. The Ho deficit in the majority of *C. mucedo* populations could therefore conceivably result from the pooling of temporally differentiated populations, inbreeding, or a combination of the two. The results of this study downplay the role of inbreeding and reinforce the hypothesis that a Wahlund effect is making a significant contribution to Ho deficits in *C. mucedo* populations. This conclusion must, however, be tempered by our small sample size, which was logistically constrained by the brief period and low incidence of sexual reproduction, and also by the difficulties in genotyping small larvae. Furthermore, as not all populations seem to undergo sex in every year (Okamura, 1997a), and of those that do, generally only a small proportion of colonies apparently produce larvae (Uotila & Jokela, 1995; Okamura, 1997b), the long-term importance of outcrossing to populations across a broad geographical area remains to be determined. Nevertheless, these findings are significant because they represent the first comparison of parental and larval genotypes in sessile, benthic freshwater invertebrates using codominant molecular markers.

Evidence for outcrossing in *C. mucedo* may provide at least a partial explanation for the unusual timing of sex in this species and, by extension, other sessile freshwater taxa with similar life histories. Key differences between these taxa and the parthenogenetic mobile zooplankton are mechanisms of fertilisation and mate selection. While zooplankton actively seek and copulate with conspecifics, sessile freshwater taxa spawn sperm into the water column that are subsequently collected for fertilisation. We suggest that an early sexual phase is a strategy that maximises rates of outcrossing within populations of sessile freshwater invertebrates (see also Okamura, 1997b) and is a result of their particular life histories. Short-lived sperm are unlikely to travel great distances, especially in the slowly moving currents of lakes and ponds, and if sperms are released early in the season, clonal proliferation will not yet have produced dense monoclonal

stands that could intercept water-borne sperm and result in self-fertilisation (Okamura, 1997b). Our postulations are consistent with the emerging body of data suggesting that inbreeding avoidance is of paramount importance in natural populations of a wide variety of animals (see Amos *et al*., 2001 and references therein).

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